Biology of the normal breast:

Relation to mammographic density and risk of breast cancer

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Series of dissertations submitted to the
Faculty of Medicine, University of Oslo
No. 1137

ISBN 978-82-8264-155-5

Cover: Inger Sandved Anfinsen.
Printed in Norway: AIT Oslo AS.

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Acknowledgements

As a medical student I new that I wanted to work with cancer, and contacted Anne-Lise Børresen-Dale to hear if I could to my master thesis with her. Ever since my first meeting with her in Trondheim in 1998, her scientific enthusiasm has strengthened my determination to work with cancer –not only in the clinical world, but also within the research community. In the same period, Gunnar Kvalheim and Jahn Nesland were the skilful and encouraging supervisors of my first scientific publication.

Stein Kvalheim gave me trust and responsibility, under skilful guidance, from day one when I started as a registrar at the Norwegian Hospital. I truly enjoyed the clinical work with my colleagues and with patients facing one of the greatest existential challenges in their lives.

These two worlds that I encountered early in my career are both important to me and to the cancer patients and I hope I can combine the two during the rest of my career.

Åslaug Helland has been my supervisor and closest co-worker during the work that has lead to this thesis. I'm grateful for her presence, involvement, realism and ability to see possibilities in the project. She also a model in the way she combines a clinical and scientific career with a family life and still keeps a calm and positive attitude.

I would like to thank Anne-Lise for accepting me as a PhD-student and for being my supervisor. Her ability to grasp a complex situation and see new aspects of it is impressive and has contributed to my learning as well as to the projects.

This project is a collaboration with many and I would like to thank all those who have been involved.

A special acknowledgement goes to Giske Ursin for the overall contribution to the project, for epidemiologic input and for receiving me at University of Southern California Keck School of Medicine.

I would like to thank Ole Christian Lingjærde for commitment to the project and invaluable statistical contributions. I have truly enjoyed his exceptional ability to make statistics understandable and interesting.

Radiologists at six different hospitals in the country have taken time in a hectic clinical day to include women to this study. I would like to thank them all for their contributions and Marit Holmen in particular for her involvement in the project from the start – and in the future.

I have collaborated with and received help from many of my colleagues at the Department of Genetics. I would particularly like to thank Hilde, Caroline and Phoung for their contribution to the MDG study, to Silje and Ole Christian for endless R support, to Vessela and Margarethe for introducing me to the world of SNPs, to Therese for feedback on my writing and to many of my colleagues for answering questions and lending an ear when I needed it. I have greatly appreciated the scientific, social and athletic environment in the lab. Thank you all!

This study would never have been if it were not for the women who participated. I am immensely grateful to, impressed by and ever indebted to all the women who participated

in the project. They gave their time, tissue and information in order to prevent breast cancer deaths in future generations.

I gratefully acknowledge the University of Oslo for admitting me into the PhD program and the Norwegian Research Counsel of Norway and to the South-Eastern Norway Regional Health Authority for the grants and financial support of the project. I would also like to thank the opponents for taking the time to read, evaluate and discuss my thesis.

I would like to thank my friends and my family. My friends and colleagues in Acem give me an interesting and rewarding extracurricular time – which yields important experience to the benefit of both work and personal life. I am particularly grateful to my parents and Elsa and Haaken: Thank you for loving our girls and for helping us out when time is scarce! Thank you, Ole, for being an excellent academic role model.

Last, but not least, I would like to try to express how much my little family means to me. Thank you, Baard, for being my best friend, my partner and the parents our daughters and for being there also in tough times! You have given me time when you've had little to give. You are my anchor and give me my needed sense of belonging. And to the most beautiful two girls in the world: Iben and Tindra. You give me joy, diversion, frustration and affection. You are what really matter!

Oslo, January, 2011

Aims

The main aim of these studies was to explore the biology of normal breast tissue. This is important in order to contribute to the creation of methods to identify women with high risk of breast cancer and early stages of the disease.

To achieve this overall aim, we decided to focus on three main topics:

1. The variation in gene expression in normal breast tissue
2. The biology underlying mammographic density, one of the strongest breast cancer risk factors.
3. The biology associated with high levels of serum estrogen.

The specific questions addressed to reach these aims were

1. Can the variation of gene expression patterns in breast tissue from healthy women be used to identify subgroups of women with different breast biology?
2. If so, what are the biological differences between such subgroups?
3. Which genes have expression levels in normal breast tissue that are associated with mammographic density?
4. Which genes have expression levels in normal breast tissue that are associated with high levels of serum estradiol?
5. Which single nucleotide polymorphisms (SNPs) are associated with mammographic density and/or serum estradiol levels?
6. Which mRNA transcripts mediate the genetic variation identified in pt 5?
7. Are the genes and SNPs identified in pt 3, 4 and 5 associated with risk for breast cancer?

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Paper I
Gene expression profiles of breast biopsies from healthy women identify a group with claudin-low features
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Paper II
Expression levels of uridine 5′-diphosphoglucuronosyltransferase genes in breast tissue from healthy women are associated with mammographic density
Vilde D Haakensen, Margarethe Biong, Ole Christian Lingjærde, Marit Muri Holmen, Jan Ole Frantzen , Ying Chen, Dina Navjord, Linda Romundstad, Torben Lüders, Ida K Bukholm, Hiroko K Solvang, Vessela N Kristensen , Giske Ursin, Anne-Lise Børresen-Dale, Åslaug Helland. Br Ca Res, 2010, Aug.

Paper III
Serum estradiol levels associated with specific gene expression patterns in normal breast tissue and in breast carcinomas
Vilde D Haakensen, Trine Bjøro, Torben Lüders, Margit Riis, Ida K Bukholm, Vessela N Kristensen, Melissa Troester, Marit Muri Homen, Giske Ursin, Anne-Lise Børresen-Dale, Åslaug Helland. Submitted to Breast Cancer Research.

Paper IV
Identification of SNP markers with putative influence on mammographic density and breast cancer risk
Biong M., Suderman M, Haakensen VD, Kulle B, Berg P, Gram IT, Dumeaux V, Ursin G, Helland Å, Børresen-Dale AL, Hallett M, Kristensen VN
Manuscript

1. Introduction

The breast cancer survival rates have improved greatly over the past decades (1969: 65%, 2008: 88%, (1)). This is partly due to earlier diagnosis and better treatment. Still, this disease is a major killer of women worldwide, with an age-standardized mortality-rate of 13% in Norway. The improvement seen on survival rates is not seen for breast cancer prevention. The breast cancer incidence continues to increase in most countries. The main reason is the poor understanding of the very first steps of breast carcinogenesis, including the complex interactions of the different risk factors for the disease (2). We use information about family history and *BRCA*-mutations to identify high-risk women, but most women developing breast cancer are not in the high-risk groups. Better identification of high-risk women will enable early diagnosis and possibly even prevention of the disease.

Breast cancer is a disease where early diagnosis improves the prognosis. Mammographic screening is used to detect the tumors early, but not all breast cancers are detected at screening. Interval cancers are diagnosed between two screening sessions. These are more often aggressive cancers with rapid growth (3) and do often occur in areas of mammographic density (MD) due to masking (the tumor is radiologically dense and a small tumor may not be visible in the dense areas) (4). Blood tests aimed at detecting breast cancer are available (5), but there is today no reliable method of detecting the very first steps of breast carcinogenesis and there is need for better tools for early detection (6).

MD is a strong risk factor for breast cancer and may be used as an intermediate to inform about breast cancer risk. The number of factors influencing such an intermediate may be fewer, producing more powerful studies (7).

The anatomy and physiology of the normal, adult breast are well described. The last decade there have been some publications focusing on the molecular biology and gene expression of the healthy breast, but much is still unknown. A better understanding of the molecular biology of the normal breast will make it easier to identify breasts that deviate from the normal on the path towards malignancy. Finding molecular subgroups of healthy breasts may help us identify high-risk groups and hence understand the molecular

mechanism involved in the development of the different breast cancer diseases.

2. Development and physiology of the normal breast

2.1 Breast development

The breast originates in ectodermal tissue forming a ridge on either side of the ventral aspect of the body from the groin to the axilla. The ridge regresses after 6 weeks of gestation, except for the areas that develop into the breasts. Accessory nipples are remnants of this ectodermal ridge that has not regressed fully. From 7 to 32 weeks of gestation, the breast develops with invasion of mammary parenchyma in the stroma, formation of epithelial buds, branching, acquisition of smooth muscle cells and formation of ducts and the nipple. During these first months, estrogen receptor (ER) is not detectable and the development occurs independently of estrogen. During the last trimester, ER is expressed and the breast tissue is estrogen sensitive (8). In this period, the level of pro-lactating hormones is high in maternal and fetal circulation, resulting in the secretion of colostrum from the breasts of some newborn infants. The breasts regress shortly after birth. Throughout childhood, the breasts remain immature and the growth is isometric (9-11).

During puberty, maturation of the breasts occurs under influence of growth hormone and estrogen. The ducts are elongated from the nipple and into the fat pads through the terminal end buds which give rise to new branches of ducts. The stroma also contributes to branching of the mammary ducts and there is a marked increase of adipose tissue in the breast. A type 1 lobule is formed and consists of alveolar buds clustered around a terminal duct (Figure 1). This is the most common lobule in nulliparous women. (10,11). As number of alveolar buds in each lobule increases, type 2 and 3 lobules will form (Figure 1), but only to a limited extent in a nulliparous breast.

The adult breast goes through cyclic changes during the menstrual cycle. In the luteal phase there is high mitotic activity and development of the lobules. In the follicular phase, the lobules are small and there is low mitotic activity.

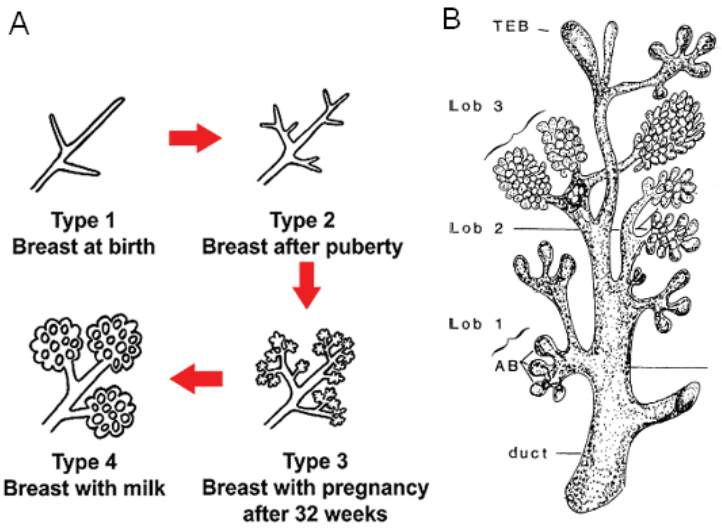


Figure 1 The lobular structures of the normal human breast. Type 1 lobules are present from birth and are most prominent in the breasts of nulliparous and postmenopausal women. A limited number of type 2 lobules forms during puberty. Type 3 lobules are formed during the last trimester. Type 4 lobules are milk-secreting. After menopause, most type 3 lobules will regress to type 1 and 2 lobules. AB: Alveolar bud. TED: Terminal end bud. A) From http://www.abortionbreastcancer.com/maturity.htm B) From Russo and Russo, 2004 (11).

During pregnancy, further elongation and branching of the ductal system and growth of the lobules is driven by female hormones and growth factors (9). As the ductal system grows, the ductules mature into acini and type 4 lobules are formed. (11). The joint action of estrogen, progesterone and prolactin are necessary for the final differentiation of the mammary gland that leads to the reduced risk for breast cancer seen after the first full-term pregnancy (12). After lactation, involution occurs, where the alveoli stop milk production and decrease in number and the ducts collapse. Until menopause, breasts of parous women still have more glandular tissue, with type 2 and 3 lobules, compared to the breasts of nulliparous women (11).

The post-lactational involution is further enhanced by menopause when the levels of estrogen and progesterone are dramatically reduced (9). During the menopausal involution, a large proportion of the type 3 lobules will regress to type 1 and 2 lobules. In

postmenopausal women, type 1 lobules are most common, both in parous and nulliparous women (11).

Interestingly, the proliferative activity (measured by Ki67-staining) is significantly higher in type 1 than in type 2 and 3 lobules (which are the most common lobules in parous women) (11). This is also the type of lobule where most breast cancers are believed to arise (13). The difference in activity between the different types of lobules is greater in nulliparous women; parity reduces the proliferative activity of the mammary epithelium. For both groups of women, breast epithelial proliferation is greatly reduced by menopause, but even for postmenopausal women, nulliparous women have a higher proliferative rate. This may explain why, in postmenopausal women, nulliparous women still have a higher risk of developing breast cancer despite the fact that both groups of women have predominantly type 1 lobules (11).

Mammary stem cell

The origin of the luminal and myoeptihelial cells has been suggested to be mammary stem cells (MaSC) (Figure 2). Stem cells divide asymmetrically and give rise to one cell identical to itself (with infinite replicative potential) and to a progenitor differentiating into the myoepithlial or luminal lineage in a hierarchical fashion (14). MaSC are able to express telomerase (15), and have an infinite replicative potential and remain in the body as active, replicating cells from embryogenesis into adult life, and do therefore have a higher risk of accumulating oncogenic alterations than other cell types (8).

The MaSc is thought to reside in the basal compartment of the epithelium in the ducts (Figure 2). Recent research has however suggested that the precursor of the two breast epithelial cell types resides in the luminal lineage (for review, see (16)). Luminal epithelial cells can, under specific conditions; become immortal and acquire myoepithelial/basal-like characteristics (17) (18). The hypothesis that MaSCs reside in the luminal lineage is supported by recent studies of breast cancer showing that luminal epithelial cells invade more intensely than basal cells and that metastatic tumors often have a luminal phenotype (CD24+) even when the primary tumor is enriched in basal-like cells (CD44+) (16,19).

MaSC are ER and PR negative (20) and two recent studies indicate that they are regulated by estrogen and progesterone through paracrine mechanisms from receptor positive neighboring cells (21,22). High levels of endogenous or exogenous estradiol and/or progesterone increased the pool of stem cells (characterized as CD49+/CD24-) and that deprivation of these hormones dramatically reduced the amount of cells counted.

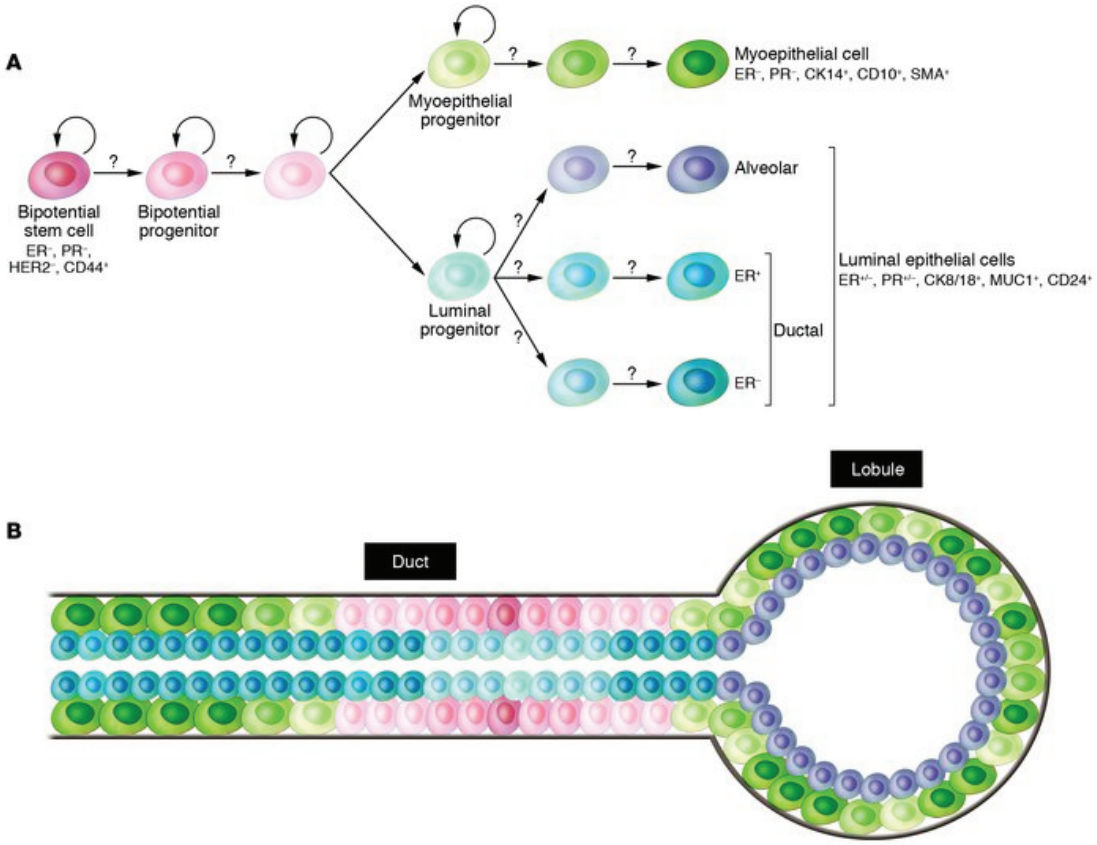


Figure 2 A) A hypothetical and simplistic illustration of the relation between the mammary stem cell (MaSC) and its progeny. B) An illustration of the normal terminal duct lobular unit. Hypothetical location of different cell types (colored as in A). The gray line is the basement membrane. CK14: cytokeratin 14; MUC1: mucin 1. From Polyak et al (23)

2.2 Anatomy of the breast

The anatomy of the adult female breast was first described by Cooper in 1840 (24) and depicted in Figure 3. The adult breast consists of 15-20 lobes each branched into lobules with 10-100 milk producing alveoli called terminal ductal lobular units (TDLUs) (Figure 1). The ducts lead the milk from the lobules to the nipple. Most breast cancers arise in the ductal epithelium. The epithelium of human breasts consists of two main cell types, present from 14 weeks of gestation and described by the staining of different keratins: Luminal epithelial cells facing the lumen of the ducts and lobules and basal/myoepithelial cells lining the basal membrane(16). In the ducts, myoepithelial cells form a continuous layer in close contact with the basement membrane and most of the communication with the stroma is mediated by these cells. In the alveoli on the other hand, the luminal epithelial cells are in direct contact with the basement membrane (25). Surface markers specific for luminal and myoepithelial cells are listed in Table 1.

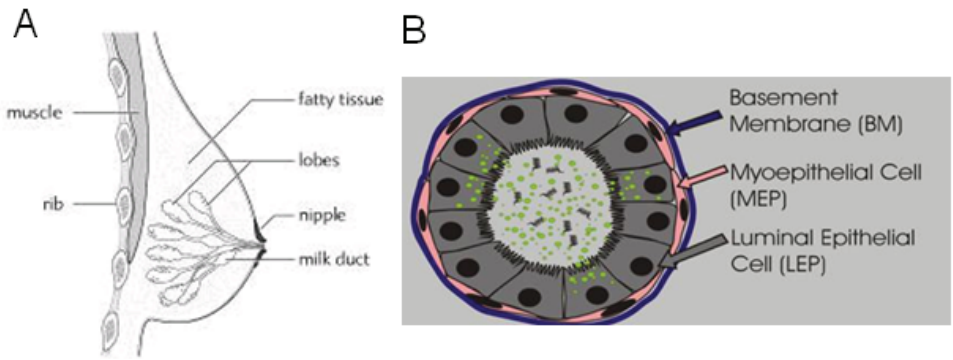


Figure 3
A) Anatomy of the human breast. From http://radonc.ucsd.edu/patientinformation/programs/breastCancer.asp
B) A cross section of the mammary duct. From Adriance et al (26).

Table 1 Gene expression markers suggested to identify different mammary cell types

Cell type	Surface marker	Gene symbol	Reference
Luminal epithelial cells	Mucin-1	*MUC1/EMA*	O'Hare et al, 1991 (27)
	B3-integrin	*ITGB3/CD61*	Asselin-Labat et al, 2007 (28)
	Cytokeratins 7, 8, 18 and 19	*K7, K8, K18, K19*	Clayton et al, 2004 (29)
Myo-epithelial cells	α-smooth muscle actin	*SMA*	Gugliotta et al, 1988 (30)
	Common acute lymphoblastic leukaemia antigen	*CALLA/CD10/MME*	O'Hare et al, 1991 (27)
	α6-integrin	*ITGA6/CD49f*	Clayton et al, 2004 (29)
	Cytokeratins 5 and 14	*K5, K14*	Clayton et al, 2004 (29)
	Vimentin	*VIM*	Clayton et al, 2004 (29)
Mammary stem cells	Cytokeratins 14 and 19	*K19 and K14* [1)]	Villadsen et al, 2007 (14)
	Cluster of differentiation 24	*CD24* [2)]	Shackleton et al, 2006 (31)
	α6-integrin	*ITGA6/CD49f* [3)]	Shackleton et al, 2006 (31)
	β1-integrin	*ITGB1/CD29* [3)]	Shackleton et al, 2006 (31)
	Aldehyde dehydrogenase 1	*ALDH1*	Ginestier et al, 2007 (32)
	B lymphoma Mo-MLV insertion region 1	*BMI1*	Liu et al, 2005 (33)
	Epithelial cell adhesion molecule/epithelial specific antigen	*EPCAM/ESA/TACSTD1*	Stingl et al, 1998 (34)

1) Co-expression of K19 and K14

2) Co-expressed with CD29 or ITGA6/CD49

3) Co-expressed with CD24

2.3 Physiology of the breast

Female steroid hormones and receptors

Estrogen and progesterone are steroid hormones produced in the ovaries. Both hormones are needed for normal breast development and function (11,35). Their receptors are localized in the nucleus and the activated receptor complexes bind to the promoter region of target genes and act as transcription factors (Figure 4). The receptor complexes also activate cytoplasmic signaling cascades.

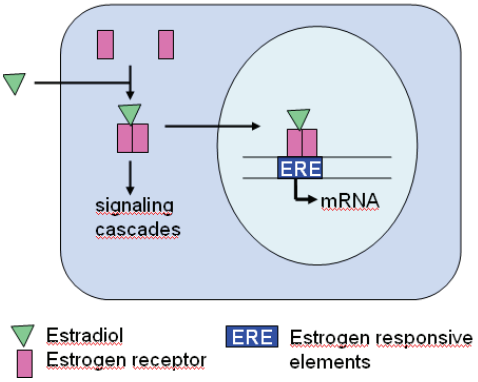


Figure 4 Estrogen binds to the estrogen receptor in the cytoplasm and cause dimerization of the receptor and translocation to the nucleus. In the nucleus the receptor complex binds to estrogen responsive elements of the DNA to induce transcription of target genes. The activated receptor complex may also induce cytoplasmic signaling cascades.

Estrogen receptor (ER) (isoforms α and β), coded by two different genes, is expressed in several tissues, including breast, endometrium, prostate and brain. The two isoforms are expressed in different cells; they regulate different genes and do sometimes oppose each other in function. The expression of ERα (but not ERβ) is down-regulated as estrogen levels increase. High expression of mammary epithelial ERα is common postmenopausally, as a response to reduced estrogen levels, and indicate non-proliferative cells (36). The two receptors are also affected differently by treatment. In breast cancer cells tamoxifen treatment increase the levels of ESRα whereas aromatase inhibitors increase the levels of ERβ(37).

Progesterone receptors (PR) (isoforms α and β) exist in several tissues, including the breast, endometrium and brain. PR is mostly induced by estrogen receptor (ER)-activated transcription in presence of epithelial growth factor (EGF), although some ER-

independent expression of PR also occurs (38). Progesterone and PR are necessary for the development and differentiation of the lobules and alveoli (TDLUs) (39). Progesterone reduce proliferation and increase apoptosis in normal breast epithelial cells and oppose the proliferative action of estrogen (12). Expression of PRα is reduced in pregnant and parous women and low levels of this receptor is suggested as a marker of reduced risk for BC (40).

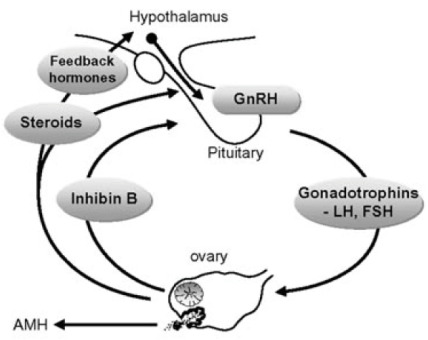
Epithelial cell proliferation

Only 7-10% of normal breast epithelial cells express ER and PR, ER is expressed to a higher degree in lobules than in ducts (41). Both receptor types are also expressed in the stromal cells (36). ERα is restricted to the nuclei of some luminal epithelial cells, but ERβ is expressed more widely (at all developmental stages of the breast) and staining is seen in the nuclei of luminal and basal epithelial cells as well as in stromal cells (both fibroblasts and endothelial cells) (42,43). The proliferating epithelial cells are not found to express ERα (44) and most often these are negative to both ER isoforms (43). In normal tissue, the estrogen-induced epithelial proliferation is, at least partly, caused by paracrine signals such as stroma-derived hepatocyte growth factor (HGF) from ER+ fibroblasts (45)

Some proliferating epithelial cells are PRβ positive and the effect of progesterone on the mammary epithelium can be both direct and paracrine through PR positive stromal cells (46). In the menstrual cycle, proliferation of the epithelial cells in the TDLU increases along with the dramatic increase in serum progesterone level in the luteal phase. The proliferative role of progesterone is also supported by animal studies showing increased proliferation of epithelial cells when estrogen and progesterone are given in combination compared with estrogen alone (38,46).

Endocrinology of menopause

Menopause is the permanent loss of ovarian function leading to cessation of menstruation (for review see (47)). Menopause is recognized one year after the last menstruation. Women are born with a fixed number of ovarian follicles that mature one for every ovulation. From the late 30s, the number of remaining follicles decline rapidly. When only about 10follicles remain, irregular menstruation will start, and at menopause few follicles are left (48). The ovarian follicles produce both estrogen and the hormone inhibin B, which inhibits secretion of follicle stimulating hormone (FSH) as part of a negative feedback-system (see Figure 5). As the number of follicles is reduced, less inhibin B is produced, which in turn leads to an increase in FSH-secretion. These alterations occur while regular menstruation is still taking place. The elevated levels of FHS ensure stable estradiol levels despite reduced number of follicles. Eventually, there is loss of ovarian function, decline in production of estradiol and progesterone and increase in both pituitary hormones FSH and luteinizing hormone (LH). In the menopausal transition, the serum levels of FSH and estradiol are varying, and diagnosing menopause on these to parameters alone is not possible (47).



Hypothalamo-pituitary ovarian axis

Figure 5 The hypothalamic regulation of gonadal sex hormones. Gonadotropin increases release of FSH and LH which stimulate ovarian secretion of sex hormones (estradiol and progesterone) and inhibins. The sex hormones and inhibins subsequently reduce the secretion of gonadotropin. The Anti-Mullerian hormone (AMH) is not involved in the feedback-loop. GnRH = gonadotropin-releasing hormone; LH =luteinizing hormone; FSH = follicle stimulating hormone; AMH=Anti-Mullerian hormone. From Burger (47).

3. Molecular characterization of normal breast tissue

3.1 Gene expression

There is extensive literature about the molecular biology of breast cancer, but a limited number of studies focusing on the molecular patterns in breasts of healthy women.

There are, a growing number of studies characterizing the gene expression patterns of normal mammary epithelium, and partly other cells in the normal breast. Studies of protein expression from single genes by immunohistochemistry, gene expression by polymerase chain reaction (PCR) or blotting in non-cancer tissues are frequent (49-51). Whole genome expression profiling of normal breast tissue is less frequent, but the last years, several studies have been performed. Most studies compare different normal breast cell types from breasts with cancerous lesions or compare normal and malignant breast tissue/cells (52-54). Some studies relate gene expression profiles of normal breast tissue/cells to other clinical features (55) or to treatment response (56).

Cell type specific gene expression profiles

The first whole genome expression profiling of different cell types from normal breast tissue and breast cancers was published by Polyaks group in 2004 (57). They isolated epithelial cells, myoepithelial cells, infiltrating lymphocytes, endothelial cells and fibroblast-enriched stroma from two mammoplasty reductions, two ductal carcinoma in situ (DCIS), 13 invasive carcinomas, a fibroadenoma and a phyllodes tumor. They used antibodies against EpCAM/ESA (epithelial cells), CD45 (lymphocytes), P1H12 (endothelial cells), CD10/CALLA/MME (myoepithelial cells) to separate the cell types. Lymphocytes were removed before isolation of myoepithelial cells to avoid contamination due to lymphocytic expression of CD10. Serial analysis of gene expression (SAGE) was used to generate cell-specific SAGE libraries and Monte Carlo analysis used to identify differentially expressed genes. They also demonstrated large differences in molecular profiles between normal and malignant cells in all cell types.

Another group has performed two studies(58,59) providing gene expression profiles characteristic of normal luminal and myoepithelial cells. Both studies used

immunomagnetically sorting of primary cultures from mammoplasty reductions to separate the two cell types with MUC1/EMA as a luminal marker and CD10/CALLA/MME as a myoepithelial marker followed by negative selection using EpCAM/ESA (epithelial cells) and integrin β4 (ITGB4) (myoepithelial cells). Their profiles were partly overlapping, and established myepithelial (LGALS7, S100A2, SPARC and CAV1) and luminal (CD24, LCN2, CLDN4, MUC1 and SEMA3B) markers were identified in both studies. These studies did not remove lymphocytes prior to isolation of myoepithelial cells. Gene ontology-terms enriched in the myoepithelial-specific gene lists do not, however, include lymphocyte-related terms, and significant lymphocyte contamination is therefore unlikely.

Two groups studied epithelial and stromal cells from mammplasty reductions and breast cancer patients after laser capture microdissection and published stromal-related gene expression profiles (60,61). Their studies provided gene expression profiles characteristic for the stromal cells compared with epithelial cells. The profiles of normal stromal cells compared with normal epithelial cells published in the two papers, overlapped with about 40%.

Polyaks group has analyzed cells from mammoplasty reductions comparing stem-like cells (CD44+) suggested to be progenitor cells compared with luminal epithelial cells (CD24+) (19). Gene expression profiles of putative progenitor cells are also published by Eaves group. They isolated and cultured primitive bipotent and luminal restricted progenitor cells and generated gene expression profiles compared to mature luminal and myoepithelial cells (62).

Such cell-specific gene expression profiles give important information about the biology of the respective cell. They also serve an important role as a comparison with gene lists generated from other studies of different cells/tissues. This study uses several of these gene lists to explore the nature of a subgroup of our normal tissue biopsies.

Subtypes of normal breast tissue

Variation in gene expression of normal breast tissue is not studied. More than a decade ago, study of variation in gene expression of breast tumors resulted in the first identification of breast cancer subtypes (63,64). Analyses of whole biopsies of breast

cancer tissue allowed an overall profile of biological features from all cell types combined. Similar to the clinical relevance of breast cancer subtypes, subtypes of normal breast tissue may be related to clinically important variables such as breast cancer risk.

Source of normal material

Mammoplasty reduction is the most widely accepted normal human breast tissue used for research. Several groups have used histologically normal tumor adjacent tissue as control in cancer-studies. The impact of the source of normal tissue has been addressed in two different studies. Finak and colleagues compared normal tissue from mammplasty reductions and from breasts with malignant disease (>2 cm away from the tumor) and found no difference (60). Different gene expression profiles between normal epithelium from mammoplasty reductions and from breasts with malignancy is, however, found in two other studies by Rosenbergs group (65,66). Graham and colleagues examined the profiles of breast tissue from prophylactically removed breasts. They found that, based on gene expression, normal epithelium from breast cancer patients and from high risk women (undergoing prophylactic mastectomy) clustered together and separate from the epithelium from mammoplasty reductions. They concluded that the shared characteristics between the cancer patients and the high-risk women cannot be a cancer-induced field effect and suggest that this is a high-risk profile (66). The method used by these two groups is similar, and is not likely to cause the divergent results. Both groups used fresh frozen samples, although the Finaks study soaked the tissue in TissueTek OCT (Somagen, Edmonton, Alberta, Canada) before storage on liquid nitrogen. Laser capture microdissection was used to isolate epithelial cells. For tumor adjacent normal samples, more than 2 cm distance to the tumor was a requirement by both groups. The controversy between these two studies indicates that further studies are needed.

3.2 Genomic alterations

Early studies of histologically normal breast tissue from cancerous breasts and of epithelial hyperplasias without atypia revealed genomic alterations interpreted to represent the initiation or early progression of breast cancer (67,68). The existence of genomic alterations in normal tissue has been confirmed in various studies.

Rosenberg's group has performed several studies comparing genomic events in normal breast tissue from reduction mammoplasties, BRCA-mutations carriers and breast cancer patients. They found genetic abnormalities in all groups of women (69). Studying the DNA of non-cancerous epithelial cells from TDLUs, there was considerably less allelic imbalance in the reduction mammoplasties (5%) compared with the breast cancer patients and *BRCA*-mutation carriers (15% and 16% respectively) (70). They also showed that the location of allelic imbalance in tumor adjacent tissue was different from that of the carcinoma and do not represent precursors of the existing cancer, but rather separate clones with possibility of future cancer development (71).

Rennstam and colleagues used high-resolution array comparative genomic hybridization (aCGH) to compare genomic alterations in prophylactic mastectomies and reduction mammoplasties and confirmed the observation of more frequent alterations in tissue from breasts of high-risk women (72). Both tissues had alterations even after removing the copy number variations (CNVs) from the analysis. The alterations found in reduction mammoplasties were generally small and represented both known polymorphic sites and regions without previously known common variants. In prophylactic mastectomies, there were more frequent alterations, and the alterations were larger in amplitude than those found in non-familial cases, and smaller than those found in carcinomas. The variation of alterations between different samples was large, both between and within individuals.

4. Breast cancer risk

The last decade has made it increasingly evident that breast cancer is a heterogeneous disease with different clinical and biological features. The division of breast cancer into estrogen receptor positive and negative tumors has been refined and the disease is now further subdivided into subtypes defined by shared gene expression patterns (63,64). Since the underlying biology and origin differ between the different breast cancer subtypes, the risk factors may also differ (73). Most studies identifying risk factors do, however, not take the different subtypes into account. Future stratification on subtypes in breast cancer risk studies may reveal new risk factors and patterns of breast cancer risk.

4.1 Epidemiologic and hormonal risk factors

Already in the 1890ies, the proliferative role of functional ovaries on the mammary gland was suggested when Beatson observed that the course of the breast cancer disease was affected by oophorectomy (74). By the 1960ies it was well established that prolonged administration of large doses of estrogens induced cancer in the breasts and other organs (75). Later, it has become clear that high serum estrogen levels are associated with increased breast cancer risk for postmenopausal women (76,77). The results are less clear for premenopausal women. Dorgan and colleagues recently found that premenopausal serum testosterone, but not estradiol, was associated with breast cancer risk (78). The hormonal influence on the mammary gland is reflected in the many hormone-related risk factors associated with breast cancer (79)(Table 2)

Early menopause and late menarche reduce the total estrogen exposure of the breast and hence the breast cancer risk (80). Pregnancy with its high levels of female hormones increases the womans risk of developing breast cancer for up to five years (81,82) possibly due to a hormonally induced increase in the number of mammary stem cells (22). During these first five years after a full term pregnancy, there is also a worse prognosis of the disease compared to breast cancer diagnosed more distant to the last pregnancy (83,84).

Table 2 Breast cancer risk factors. Strength indicates the association between the risk factor and breast cancer in terms of relative risk. From Trichopoulos et al, 2008 (79).

Risk factor	Category/change	Strength[1)]
Gender	Women vs men	++++
Age	Increase	++++
Ethnic group	Caucasion vs Asian	+++
Family history	Yes vs no	+++
Specific genes	Yes vs no	++++
Cancer in other breast	Yes vs no	+++
Height	Increase	++
Postmenopausal obesity	Increase	++
Brith weight	Increase	+
Having been breastfed	No vs yes	0
Growth in early life	Increase	+
Atypical hyperplasia	Present vs absent	+++
Mammographic density	High vs low density	+++
Age at menarche	Earlier	
Age at menopause	Later	
Type of menopause	Natural vs artificial	++
Age at 1st full term pregnancy	Later	+++
Age at other pregnancies	Later	+
Parity overall	Lower	++
Pregnancy timing	Proximal vs distant	+
Lactation	No vs yes	+
Abortion	No vs yes	0
Oral contraceptive use (recent)	Increase	+
Hormone replacement	Increase	+
Plant foods and olive oil	Reduced intake	+
Saturated fat	Increased intake	+
Physical activity	Reduced	+
Ethanol intake	Increase	+
Ionizing radiation	Increased	+
Magnetic fields	Increased	0
Organochlorines	Increased	0

1) Associations: ++++ very strong, +++ strong, ++modest, + weak, 0 null.

Despite the initial increase in risk, higher parity is protective, the protection starting from 1years after giving birth. The protection lasts throughout the women's lifetime, with the

greatest effect going from zero to one full term pregnancy (85). Early age at first full term pregnancy and breast feeding also protect against breast cancer (80,86,87). Early age at first full term pregnancy is particularly preventive for ER+/PR+ breast cancers (88). Despite the protective effect of parity, several studies have concluded that high age at first or last full term pregnancy confers a higher risk for breast cancer than nulliparity (89,90).

The biological mechanisms underlying the different effects of pregnancies on breast cancer risk are largely unknown, but the role of female hormones is essential in all hypotheses. Several groups are even trying to develop preventive treatments trying to mimic pregnancies (85).

Hormone therapy after menopause is associated with increased risk for breast cancer, especially seen for combined estrogen-progesterone regimens (91,92). This is true for receptor positive, but not receptor negative breast cancers (88) and for both ductal and lobular hitologies (88). Anti-estrogen treatment (Tamoxifen) is associated with reduced risk of breast cancer (93,94). The association with ER+ cancers is consistent with findings that current hormone therapy use at time of diagnosis is associated with good prognosis of the breast tumor and hormone use is therefore suggested to induce breast cancers with a less aggressive phenotype (95,96). Progesterone therapy is associated with an increase in MD (97-99), increased apoptotic rate, differentiation and proliferation of epithelial cells. The effects do, however, vary between different progestins (100).

Other hormones have also been related to breast cancer risk. A recent meta-analysis found that high serum IGF1 levels increase the risk for ER+ breast cancer. There are indications that the GH/IGF1-axis contributes to hyperplasia and carcinogenesis (101,102). Reduction of IGF1-production by growth hormone antagonists reduced breast cancer development in mice and the protein has been proposed a target for prophylactic treatment (103).

Body mass index (BMI) has been found to be associated with breast cancer inversely in premenopausal women and positively in postmenopausal women (104). The association between BMI and breast cancer also varies with race and hormone receptor status such

that high recent BMI increases the risk of receptor-positive breast tumors especially in postmenopausal African-American women (73,105). BMI is a result of both genetic and environmental factors. Although there are consistent findings on the association between BMI and breast cancer risk, there are more uncertainties regarding diet.

A large meta-analysis recently confirmed that diet has a small, but significant effect on breast cancer risk. A prudent diet decreases the risk and high alcohol intake increases the risk. In this meta-analysis, a prudent diet generally consisted of large amounts of plant foods and low amounts of red and processed meat. They do, however, point out the evident error of misclassification of diets as detailed information of individual foods could not be included in the pooled analysis. The slight effect of diet could therefore be due to classification errors (106).

Physical activity reduces the risk for breast cancer, especially for postmenopausal women. A recent review found a risk reduction raging from 20-80% in different studies and a trend analysis indicating a 6% reduction in breast cancer risk pr weekly hour exercise (107). Reduced risk for breast cancer by physical activity is also shown experimentally in animal models (108). The underlying mechanisms are unknown. Suggested mechanisms are reduced levels of sex hormones and IGF1 and prevention of overweight. A recent study found that aerobic exercise reduced the non-dense breast tissue relative to the reduction BMI, but the MD was not significantly altered. They suggested that the mechanisms for the protective effect of exercise go through other mechanisms than MD (109). Alteration in serum-levels of several biomarkers from physical activity is shown. Amongst the alterations seen were reduced levels of IGF1, growth hormone (GH), tumor necrosis factor alpha (TNFα), leptin and estrogen. This was interpreted as an alteration in glucose homeostasis and metabolism (108).

There is evidence that environmental factors affect breast cancer risk already from the fetal life on. The role of birth weight in breast cancer etiology is reviewed by Michels and Xue (110). They concluded that high birth weight was associated with premenopausal, but not postmenopausal breast cancer. This is thought to be caused, at least partly, by elevated levels of growth factors leading to an increased number of mammary stem cells (110). This is supported by recent findings that women with a birth weight above 4 kg

had a 3 fold risk of developing high MD compared to women weighing 3-3.5 kg at birth (111). Large initial weight loss after birth, rapid growth in early life as well as growth patterns during adolescence are also associated with increased risk of breast cancer (112,113).

The group of Trichopoulos has proposed a model explaining the mechanisms underlying breast cancer development grouping the different risk factors according to their etiologic explanations. They outline four main mechanisms contributing to breast carcinogenensis, each associated with specific risk factors, see Table 3 (79). This gives a broad overview of underlying physiology, but does not include molecular mechanisms which will be reviewed in a separate chapter.

Table 3 Four main mechanisms contributing to breast carcinogenesis and associated specific risk factors. From Trichopoulos, 2008 (79).

General principles of carcinogenesis	Number of mammary stem cells	Growth enhancing mammotropic hormones	Terminal differentiation of the ductal tree
Age	Mammographic density	Gender	Age at 1st full term pregnancy
Ionizing radiation	Atypical hyperplasia	Age incidence pattern	Age at other pregnancies
Family history	Gender	Age at menarche	Parity overall
Specific genes	Birth weight	Age at menopause	(Lactation)
	Growth in early life	Oral contraceptives	
	Height	Hormone replacement	
	Ethnic group	Pregnancy timing	
		Postmenopausal obesity	
		Ethanol intake	
		Physical activity	
		Adult life diet	

4.2 Mammographic density

Mammograms are x-ray images where fat is represented as dark/lucent areas and epithelial and stromal tissues are represented as light/dense areas (114)(see Figure 7).

Methods of determining mammographic density

Several methods of estimating breast cancer risk from mammographic features have been introduced. In 1976, Wolfe presented a classification of breast parenchymal patterns into

four classes (115). The low-risk group, N1, was described as primarily fatty tissue. Two medium-risk groups, P1 and P2, had <25% or >25% prominent ducts respectively. The high-risk group, DY, had mammograms consisting of dense fibroglandular tissue and was estimated to have 37 times higher risk of future breast cancer development, an estimate that would later be proven too strong (116). An alternative classification of breast parenchymal patterns was introduced by Tabar (117). A combination of anatomical and mammographic features was used to subdivide the women into 5 groups. Patterns I to III were low-risk groups and patterns IV and V consisted of dense tissue of different character and were considered high-risk groups.

Quantitative estimation of percent density was first introduced by Wolfe (118). The American College of Radiology has, as part of its Breast Imaging Reporting and Data System (BI-RADS), developed a quantitative classification system of breast tissue densities that is included in clinical practice together with qualitative, descriptive methods (119). Semi-computerized quantitative methods have been developed (120,121). The qualitative methods add little information to the quantitative, and the introduction of digital mammography has made the semi-computerized method suitable for clinical use and screening programs (122). The inter-observer agreement in quantitative methods has been higher than that of qualitative method (123). During the last years, the use of MRI to measure water content of the breast as a marker of fibroglandular tissue (124) and volumetric breast density (125) have increased. The correlation between glandular tissue as measured by MRI and percent mammographic density is low, particularly for breasts with high density (126).

There is no consensus as to which estimate for mammographic density should be used. The use of qualitative techniques is still widespread; particularly the use of BI-RADS in the US both for diagnostic and research purposes. In the recent literature, many studies use both absolute and percent density, while others use only percent mammographic density. A recent study concluded that absolute density predicts breast cancer risk better than percent mammographic density (127).

High MD is a well established risk factor for breast cancer, with an increased risk of 4-6 even after correcting for other known risk factors (128-130) and regardless of breast

cancer subtype (131). The increase in breast cancer risk by using hormone therapy after menopause is greater in women who have dense breasts (132). Unlike the use of hormone therapy, a high MD increase the risk both for ER+ and ER- breast cancer (133) and is associated with both luminal A and triple negative disease (131). However, a recent Danish study found that the breast tumors developed in dense breasts are on average, less aggressive than those developed in predominantly fatty breasts (dividing all breasts into dense or fatty). The overall mortality was still higher in women with dense breasts (134). High MD may also conceal a small tumor and a measure of MD can therefore also be a sign of the sensitivity of the mammogram as a diagnostic test (4).

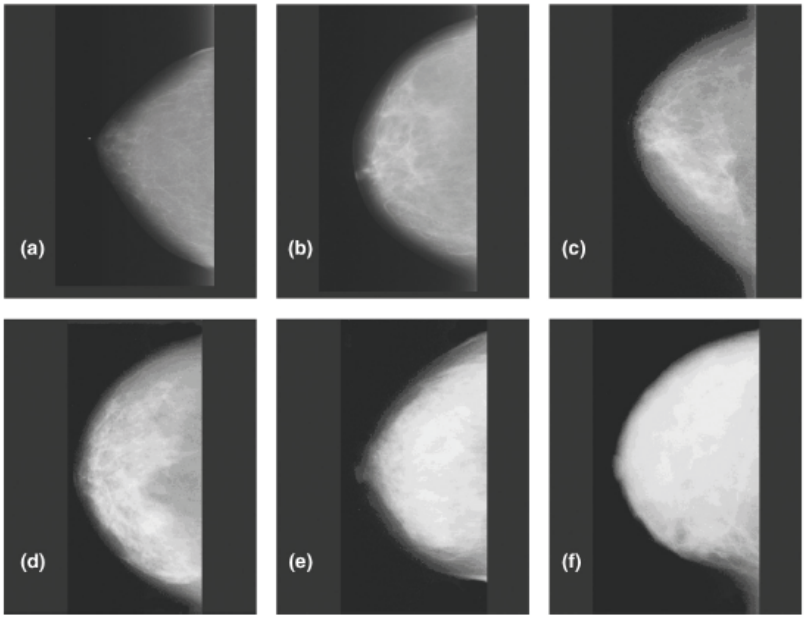


Figure 6 Percent mammographic density: (a) 0, (b) <10%, (c) 10-25%, (d) 26-50%, (e) 51-75%, (f) >75%. From Yaffe et al (125).

The biological basis of mammographic density

Breasts with high MD have a larger proportion of white/dense areas on the mammogram (Figure 6) that represent both epithelial cells and stromal components, such as collagen

and fibrosis (135-137). MD is associated with the relative area occupied by collagen, glandular structures and nuclei (of both epithelial and non-epithelial cells) (137). One group found the number of epithelial cells to be greatly increased in areas of high MD, but found no increase in proliferation as measured by the proliferation marker mindbomb homolog 1 (MIB1) (138). High MD is heterogeneous at the histopathologic level and may reflect both tissue with few cells but rich in collagen and fibroglandular tissue with high cellular activity. There may be different biological processes underlying high MD in these varying situations (139). To approach this problem, visual inspection of the mammogram can be used to distinguish between glandular and sheetlike structures of the densities. MRI may also allow specification of which type of dense tissue to measure, as demonstrated by Klifa and others (126).

Some possible mechanisms for the influence of high MD on breast cancer risk are suggested. Firstly, abundant and aberrantly activated fibroblasts may influence epithelial cells through secretion of growth factors and chemokines. Vachon and colleagues found increased aromatase in stroma and epithelium of dense areas of the breast compared with non-dense areas of healthy women. This may lead to higher estrogen-stimulation of proliferation and contribute to the carcinogenic process (140). Secondly, increased collagen deposition due to excessive fibroblast activity results in a stiffer extracellular matrix which has been associated with altered cell signaling and increased epithelial cell proliferation. Evidence supporting this view has come from two different groups using different approaches. Provenzano and colleagues found that increased collagen promoted proliferation and invasion of epithelial cells in the absence of fibroblasts (141) and that increased stromal collagen increased tumor formation and invasion significantly (142). Similarly Weavers group induced collagen-crosslinking which was accompanied by increased focal adhesion and invasion by oncogenic epithelium (143).

Still, much is unclear regarding the regulation of mammographic density and its role in breast carcinogenesis. In a recent interview, Valerie Weaver said that “my belief is that all folks who claim that they are modeling breast density when they study the effect of increased collagen concentration on cell behavior ex vivo are overinterpreting and extending data that are not yet conclusive.”(144) Studies on human tissue from a relevant

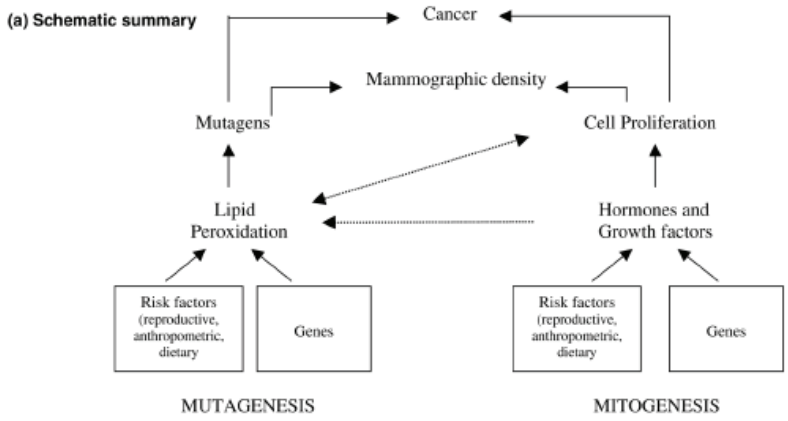
study population would help interpret in vitro studies and generate more relevant and specific hypotheses.

Epidemiologic factors and mammographic density

The regulators of MD and the impact of MD on breast carcinogenesis was recently reviewed by Martin et al (129). The genetic component of MD is substantial, and probably account for more than 50% of the variation (145) (146), but environmental factors are also important (145). MD is inversely associated with age, BMI and smoking. Menopause reduces the proliferative stimulation of the breast and hence MD. High BMI with a high fatty component of the breast is associated with reduced MD as adipose tissue is radiolucent. Smoking is suggested to have an anti-estrogen effect on the breast tissue (147,148). As mentioned previously, it was recently found that high birth weight is also associated with high MD in later life (111). This study does, however, not address whether birth weight is predominantly genetically or environmentally determined.

Hormones and mammographic density

Although exposure to endogenous female hormones is associated with proliferation of breast epithelial cells and with an increase in breast cancer risk (149), there is some controversy as to weather such exposure increases MD (150,151). Hormone therapy does, however, increase MD while anti-estrogen substances reduce MD (152)(for review, see Martin et al (129)). There is evidence that sex hormones and MD both independently increase the risk for breast cancer and that the underlying mechanisms are unrelated (153). Boyd and colleagues(154) have proposed a model for the relation between the mechanisms underlying MD and how they relate to breast cancer (Figure 7).



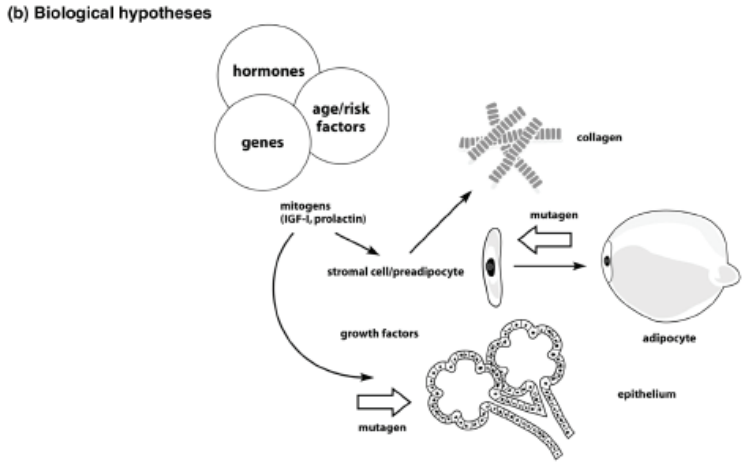


Figure 7 Mechanisms underlying mammographic density and its relation to breast cancer risk depicted by Norman Boyd and colleagues (154). Risk factors and genetic variants influence the two suggested mechanisms; mutagenesis and mitogenesis. Increased oxidative stress with lipid peroxidation increases the risk for mutations and accumulation of events that increase proliferation, stromal activity and carcinogenic drive. Hormones and growth factors stimulate stromal production of collagen and cell proliferation important both in MD and carcinogenesis. A) Scematic summary of suggested mechanisms. B) The biological hypotheses where each cell in the breast influence its neighbors. Fibroblasts produce collagen, paracrine factors influencing epithelial cells and may differentiate into adipocytes. Epithelial cells and fibroblasts proliferate upon hormonal stimuli and could initiate the carcinogenic process.

Genetic variation and mammographic density

Single nucleotide polymorphisms (SNPs) associated with MD have been identified in several genes, including *COMT*, a gene coding for an enzyme inactivating estrogens, in *ESR1* (reviewed in Kelemen et al (7)) and in and *HSD3B1*, also involved in the sex steroid metabolism. Some of these and other SNPs in the estrogen pathway were tested in a recent study, but the association with MD could not be confirmed (155). In the same year, Yong and colleagues found SNPs in the sex hormone metabolizing genes *SULT1A1* and *UGT1A* to be associated with MD. SNPs in *IGF1* and its related genes have also been linked to MD and to the serum levels of IGF1 which is in itself related to MD (153,156). Since MD is a strong risk factor for breast cancer, it was expected that SNPs associated with breast cancer risk might be associated with MD. This is so far not found (157).

Molecular variation according to mammographic density

The molecular background for MD has been explored in several studies, and gene expression alterations associated with high MD have been described (55,136). Yang et al analyzed histologically normal tissue from breasts harboring breast cancer sampled during surgery. Women were divided into high or low MD by BI-RADS. They found 73 genes differentially expressed between women with high and low MD, with a decreased transforming growth factor beta (TGFβ)-signaling in breasts with high MD (55).

Looking at regional differences within the breast, the expression of the proteoglycans lumican and decorin were found increased in regions of the breasts with high MD compared with low-MD regions, evaluated by immunohistochemistry. These proteoglycans are expressed in the stroma and have previously been reported differentially expressed between tumor and normal breast tissue (158). The expression of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in breasts have been analyzed for association with MD, but no significance was found (159).

In summary, it is well known that MD confers an increased risk of breast cancer, but the underlying mechanisms are still unclear. Specifically, it is not known whether the increased risk for breast cancer due to high MD is caused by the increased amount of cells at risk of developing cancer or due to altered biological processes. MD seems to represent presence of both stroma and epithelium, but is not influenced by the

proliferation rate per se. The histologically heterogeneous nature of high MD supports a hypothesis that different biological mechanisms lead to MD. Epidemiologic factors associated with MD have been identified, but how they are linked with MD at the cellular/molecular level is not known. The first evidence of SNPs and transcripts with putative association with MD has emerged, but much is still to be elucidated before we can identify the mechanisms underlying high MD and its association with breast cancer which may eventually allow identification of high-risk individuals in order to introduce preventive strategies. This was the main reasons for initiating the current study.

4.3 Molecular alterations associated with breast cancer risk

Genetic

The strongest genetic factors affecting the risk of developing breast cancer are mutations in the DNA-repair genes BRCA1 and BRCA2 (160,161). The inheritance of one mutated allele confers a life-time risk of up to 80% of developing breast cancer (162). Inactivation of the wild-type allele leads to defect repair of DNA and increased genetic instability and risk for cancer development. Other tumor suppressor genes were inherited mutations lead to increased risk of breast cancer are known, such as FANCJ and FANCN (Fanconi anemia), TP53 (Li Fraumeni syndrome), PTEN (Cowden syndrome), STK11 (Peutz-Jeughers syndrome) and CDH1.

Most breast cancer cases are not due to a known germ line mutation. Acquired genetic and epigenetic alterations are thought to be caused by complex interactions of genetic predispositions and environmental factors. There is a familial clustering of the disease independent of epidemiologic factors, supporting the hypothesis of a genetic component in the development for sporadic cases. Most women with first degree relatives with breast cancer will never get the disease (163).

A multigene model including common gene variants with lower penetrance most likely explains familial relative risk observed and several genome-wide association studies have been performed to identify such polymorphisms (for review, see (164) and (165)). Where previous studies focused on genes known to be involved in cancer-related biological processes (candidate gene studies) the genome-wide association studies (GWAS)

examines the whole genome to identify genetic variants and combinations of such that are associated with the disease. ATM and CHEK2 are examples of genes where medium penetrance polymorphisms have been identified. It is estimated that twelve candidate susceptibility SNPs identified explain 5-8% of the familial clustering of breast cancer, indicating that much of the underlying biology is still unknown (164,165).

The Breast Cancer Association Consortium (BCAC) is a forum created to investigate the heritability of breast cancer. In this forum, researchers from all over the world combine studies from different groups to get reliable data to evaluate the contribution to breast cancer risk from SNPs. Their meta-analyses have identified novel SNPs (166) and have confirmed some (167), but not all SNPs previously suggested (168). Some of the SNPs identified were associated with specific histopathologic subtypes (169).

Recently, pathway analysis has been introduced as a means of identifying genetic associations to breast cancer risk with the underlying assumption that different genes may affect the same pathway and result the same biological consequences for the cell (170). The pathway approach identified a significant association between the estradiol metabolic pathway (including *CYP19A1* and *UGT2B4*) and breast cancer risk (171).

The search for mediators of the identified genetic variants is also ongoing. One approach as been to use SNPs identified to be associated with breast cancer and look for difference in association between the SNP and breast cancer development according to established risk factors for the disease. Recently, two studies used this approach, one with a negative result (172) and one found an association between a SNP in MAP3K1 and height (173). This indicates that the mediation of the risk conferred by the SNPs identified is complex.

Gene expression

Little is known about gene expression profiles in normal breast tissue with increased risk for breast cancer. Partly, this is because true normal breast biopsies are not easily obtained.

One breast cancer gene expression risk signature is published (174). Chen and coworkers used histologically normal tissue and tumor tissue from the same breasts . The basic assumption was that normal tissue with tumor-like gene expression has higher risk of

developing breast caner. The genes from the histologically normal tissue whose expression was correlated with that in the tumor were included in the malignancy risk signature. This gene list was dominated by proliferative genes.

4.4 Risk prediction tools

Estimation of the breast cancer risk of individual women is important to determine who should have more frequent examinations and who should receive preventive measures. Several models assess the risk of breast cancer or the likelihood of finding a BRCA mutation or both (111) (for review, see Amir et al (175)). While some models mainly focus on family history, others take hormonal factors into account. They find that although some of the models are well calibrated to their target populations, all models have only moderate accuracy and most only include a small subset of known risk factors (175,176). I the review by Amir et al, none of the models evaluated included MD despite the strong correlation with risk and the high reproducibility (177). MD has, however, been incorporated in a few risk prediction models (including the Gail model) with a modest increase in discriminatory power (178-181).

The heterogeneous nature of breast cancer is probably reflected in different carcinogenic processes and different importance of risk factors. An example is how BRCA1 mutations tend to give basal-like breast cancers, whereas BRCA2 mutations tend to give luminal breast cancer. Risk assessment studies stratifying for subtype may reveal new knowledge of the interplay of different risk factors in the carcinogenic process (182). In the mean time, commercial genetic risk tests including breast cancer risk are being offered to consumers over internet (eg: 23andme, Navigenics and deCODE Genetics) and to clinicians (Intergenetics). These kits are considered medical devices, and the producers have recently received information that they require approval by the US Food and Drug Administration for marketing (183). Most risk prediction tools are better at a population level and the use of the tests currently available for individuals is controversial (184).

5. Breast cancer development and progression

Breast cancer is thought to originate in one cell that is transformed from a normal epithelial cell to a breast cancer cell. Carcinogenesis is a multistep process affecting the cells genome. In order for a cell to become malignant, it must acquire the characteristics nicely described by Hanahan and Weinberg in 2000 as the hallmarks of cancer (185). The present study does not focus on breast carcinogenesis in itself, but aims at understanding the normal biology in order to be able to identify the first deviating steps in the carcinogenic path. In paper I, we have identified a group of samples that share certain features with stem cells, stromal cells and partly with myoepithelial and mesenchymal cell. The role of these cell types in carcinogenesis will therefore be briefly reviewed.

5.1 Cancer stem cells or clonal evolution?

One characteristic feature of most cancers is the cellular heterogeneity within each tumor. This heterogeneity makes it hard to hit all cancer cells by the same treatment. Studies of the difference between the cells of a tumor may reveal its history. The mechanisms behind this heterogeneity are debated. The two main theories are the clonal evolution model and the cancer stem cell hypothesis (for reviews, see (186-188)).

The cancer stem cell hypothesis (hierarchical model) suggest that the cancer arise in stem cells that acquire malignant potential through a multistep carcinogenic process and that cancer stem cells further differentiate to form the different cancer cells constituting the heterogeneity of the tumor. There is a hierarchical nature where the pluripotent cancer stem cells differentiate into lineage restricted non-tumorigenic cancer cells populating the tumor. These lineage restricted cells have a limited life span, and are replaced by the cancer stem cells other progeny. The cancer stem cells are thought to be responsible for invasion and metastasis (186) and they are believed to be resistant towards chemotherapy (189).

The origin of cancer stem cells is debated. This debate is partly fueled by results indicating that other cells may acquire stem cell-like characteristics (190), in the breast illustrated by epithelial cells acquiring stem cell traits after epithelial-mesenchymal

transition (191). Initiation of tumor development has also been attributed to cancer cells lacking stem cell characteristics, raising concerns about the validity of the stem cell hypothesis (192). And there is evidence for separate evolution of CD24+ and CD44+ cells existing in the same tumor (193). The uncertain origin is reflected in the multitude of names given to the cells; Cancer stem cells, stem-like cancer cells and tumor initiating cells.

The model of clonal evolution (stochastic model) closely resembles the model of evolution of species and was first proposed by Nowell in 1976 (194). This model suggests that cancer arises in a normal cell through a multistep carcinogenic process. The cancer cell will continue to divide. The carcinogenic process has rendered the genome unstable and new genetic alterations will occur that mark the start of a new clone. The tumor cell population is the result of an evolutionary process with selection of the fittest cell clones (186,187,195).

5.2 The role of the microenvironment

The microenvironment of the breast is generally thought of as all breast components other than the epithelial cells or tumor cells, the most important being the stromal cells (mainly fibroblasts, endothelial cells and immune cells), blood and lymph vessels and extracellular matrix. Already in 1973, there was a publication showing how stromal tissues influenced proliferation of the mammary gland in a mouse model (196). The importance of the microenvironment in cancer was suggested from the early 1980s by the Bissell lab (197) and by Dvorak who compared cancer with wounds that do not heal (198). The central role in initiation and progression of the disease has only been widely accepted the last decade (for reviews, see (199-201)). Even Dvorak's comparison with wound healing has gained support in recent years by a study showing activation of host wound responses in the microenvironment of breast cancers (202).

Parallel with the malignant transformation of the luminal epithelial cells, the stroma undergoes morphological changes such as increased number of fibroblasts and lymphocytes, angiogenesis and remodeling of the extracellular matrix. It has become evident that the stroma not only responds to epithelial changes in breast cancer

progression, but have an active role in promoting cancer development and even have initial genetic alterations and that the communication between the epithelial cells and the stroma is bidirectional (200,203-206).

Several mechanisms by which the stroma can initiate malignant transformation have been proposed. Alterations in the stroma leading to a phenotype promoting a malignant transformation can be induced by carcinogens, altered expression of matrix metalloproteinases, immune cells and viruses (206). Transdifferentiation of other cell types has been suggested as a possible mechanism, with possible cells of origin being circulating fibrocytes, bone-marrow derived mesenchymal stem cells and endothelial cells going through mesenchymal transition (201). The importance of the stroma in epithelial carcinogenesis corresponds well with the role of stromal cells in regulating epithelial cell proliferation as discussed in section 2.3.

The stroma also defines the stem cell niche which regulates the stem cells. According to the cancer stem cell hypothesis, the first malignant cell is a stem cell. This is supported by evidence that dormant stem cells may be activated by changes in the local microenvironment, leading to cell fusion and cancer initiation (207) and by studies indicating that the extracellular matrix has a role in regulating tumor evolution through the stem cell niche (188).

5.3 Myoepithelial cells

Myoepithlial cells are localized between the luminal epithelial cells and the stroma and form a barrier for the cancer cells during carcinogenesis (Figure 3) (208-210). Their role as natural tumor suppressors with importance in the early stages of tumorigenesis has been confirmed in molecular studies. The extensive molecular characterization of breast cells performed by Allinen and colleagues showed that the cell type with the largest and most consistent alterations between normal tissue and both DCIS and invasive cancer was the myoepithelial cells (57). Further loss of function of these cells is suggested as the initiating event in the transition from in situ to invasive cancer (211).

5.4 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) describes a process where differentiated epithelial cells loose epithelial characteristics such as polarity and adherence to neighboring cells and tissues and gain immature, mesenchymal characterstics such as a loss of polarity and migratory and invasive properties. These properties are also important in cancer progression and metastasis. The process is regulated by and can be induced by TGFβ (212).

Several groups have shown that induction of EMT results in the gain of stem cell-like characteristics indicating a role of EMT in carcinogenesis (191,213,214). The combined features of EMT and stem-like characteristics are also seen in residual breast cancers after conventional chemotherapy and in the claudin-low subtype and is associated with bad prognosis (215,216).

6. Material and methods

6.1 Subjects

Women participating tin the present study were included from 2002 to 2007. The inclusion was done by radiologists at breast diagnostic centers. Breast diagnostic centres in six hospitals in Norway included patients to the study. The six hospitals are Oslo University Hospital Radiumhospitalet, University Hospital of North Norway, Vestfold Hospital, Innlandet Hospital, Buskerud Hospital and Sørlandet Hospital. Two groups of women were eligible: 1) Women with mammographically normal breasts (with no signs of malignancy) and at least one area with some of mammographic density (healthy women) and 2) Newly diagnosed breast cancer patients before any treatment. In total, 186 women were included, 120 healthy and 66 breast cancer patients. All women were above the age of 20 and signed informed consent. Exclusion criteria were breast implants, anticoagulant therapy, current pregnancy or lactation. Women with a history of breast cancer and no suspicion of malignancy could be included in the group of healthy women with a biopsy of the contralateral breast. The study was approved by the ethical committee (S-02036).

Table 4 Referral to the breast diagnostic centre of women included in the study

Referral	Total	Healthy women	Breast cancer patients
	n (%)	n (%)	n (%)
Screening	69 (37)	50 (42)	19 (29)
Findings/risk [1)]	83 (45)	41 (34)	42 (64)
Unknown	34 (18)	29 (24)	5 (7)
	186 (100)	120 (100)	66 (100)

1) Palpable lump/clinical findings/increased risk of breast cancer (family history or previous benign breast lesion or breast cancer).

The women were referred to the breast diagnostic center in various ways (see Table 4 and Figure 9). Most women were referred from their doctor due to a palpable lump, clinical finding or high risk (family history or previous breast cancer or benign breast lesion). A

total of 69 women were referred from the National Breast Cancer Screening Program (217) due to irregular findings. Each woman provided two breast biopsies, blood samples, mammograms and filled in a questionnaire about parity, hormone use and family history of breast cancer.

6.2 Core biopsies

From each woman two biopsies were collected by use of a 14 gauge needle. Biopsies from Oslo University Hospital Radiumhospitalet were snap frozen in liquid nitrogen and stored at -80°C. In the other hospitals one biopsy from each woman was soaked in RNAlater (for RNA-extraction) and another on 70% ethanol (for DNA-extraction). These biopsies were transported to Oslo University Hospital Radiumhospitalet, Deptartment of Genetics, and stored at -20°C until extraction.

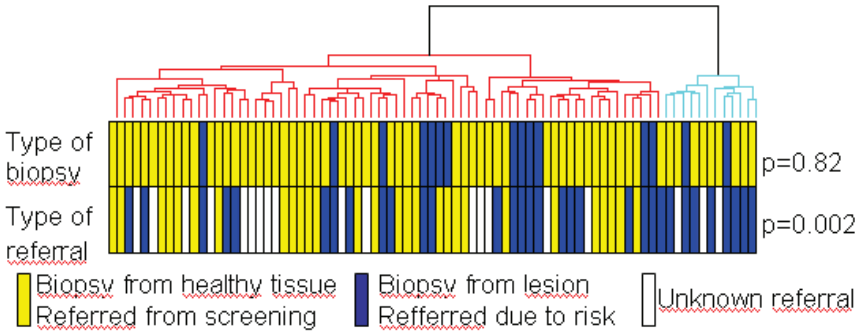


Figure 8 Unsupervised hierarchical clustering of gene expression from 79 healthy women (9767 probes). Biopsies taken from lesions do not consistently cluster together, and are not significantly enriched in any of the main clusters. Source of referral is significantly different in the two main clusters. P-values from ANOVA-tests.

The biopsies from the breasts of healthy women were taken from an area with some mammographic density in order to avoid purely fatty biopsies. One hospital (including 17 healthy women) sampled biopsies from the benign lesion/suspect area and not from normal tissue with some density. These included 5 fibroadenomas, 8 fibroadnomatous breasts, one microcalcification and three without mammographic lesions (biopsy was taken from a palpable structure not found on mammography). These 17 samples did,

however, not consistently cluster together in unsupervised, hierarchical clustering and there is no significant difference in biopsies taken from a lesion as opposed to from healthy tissue in the two main clusters observed (see Figure 8). In breast cancer patients, the biopsies were taken from the tumor itself.

6.3 Whole genome expression analysis

DNA microarrays are developed to perform whole-genome analyses of DNA-level or mRNA-expression. Several different types of microarrays are used. We have used two-colour olignucleotide microarrays with printed 60-mer probes. On each array, nucleotide sequences of 6base pairs are printed on separate spots. A common reference is used for all experiments. We used a commercially available reference prepared from 10 different cell lines to obtain a general background for our test samples. Separate labelling is used for the test and reference RNA before they are mixed into one solution and dispensed onto the slide.

RNA from the test and reference samples will hybridize competitively to the printed nucleotide sequences and the relative expression of each mRNA transcript can be determined from the scanned ratio of signalling from the two dyes. The array will not give information about the exact level of expression of the particular probe, but rather the expression level in the sample compared with the control for each probe. The protocol used in these studies is described in more detail in the following, and is illustrated in Figure 9. For more detailed description of the experiments in this study, see paper II.

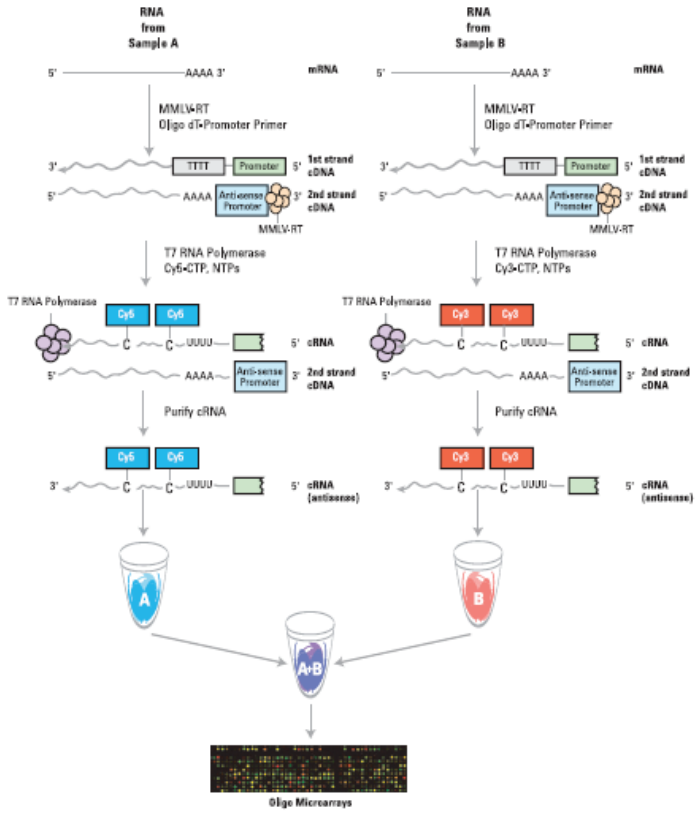


Figure 9 Fluorescent cRNA Synthesis Procedure illustrated in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Protocol, Version 2.0. August 2003.

6.4 RNA data processing

Normalization

After hybridization, the microarray slide is scanned and the colour intensities are converted into numbers. In a two-channel RNA-microarray experiment, the output value for each spot is typically the log2-ratio of red (test-sample) over green (control) signal intensity. There may be differences in signal intensity between probes on one array leading to technical bias in the experiment. Both labelling efficiency and scanning

properties may influence the relative intensity of each dye. Within-array normalization is a set of techniques to correct for these technical biases. A typical technical bias is different hybridization intensity for the two dyes used in the experiment. This will lead to a non-linear relationship between spots dominated by each colour. This can be illustrated by an MA-plot. A standard, normalized measure for the intensity in a spot is given by M=log2R-log2G, where R=red intensity and G=Green intensity in a spot. A measure of the overall brightness of a spot is given by A=(log2R+log2G)/2. These two measures, A and M, are used to visualize the distributions of the intensities on an array and in a dataset in the MA-plot. In Figure 10A), we see an MA plot representing a non-normalized microarray experiment. The banana-shape of the distribution of intensities indicates a non-linearity. After normalization, the same data has become linear and the distribution of red and green intensities is more equally represented on the array B)(218,219).

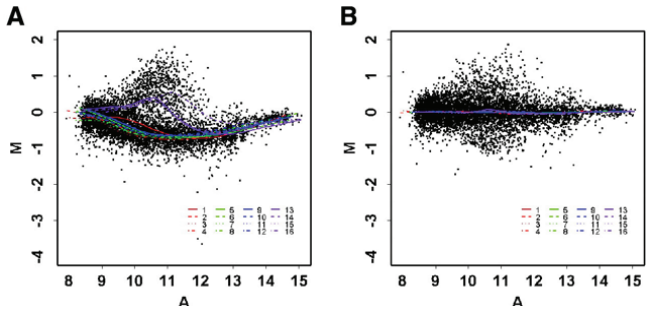


Figure 10 MA-plot with A) non-normalized data and B) normalized data. From Yang Y H et al, 2002 (218).

Locally weighted scatterplot smoothing (Lowess) is a linear regression method that is commonly used for normalization of two-channel microarray data (220). The method adjusts for dye-biases within one array. This occurs because the dyes do not fluoresce at the same intensity at different levels. Lowess-normalization ensures that the log2-ratio-intensities achieved from all spots in one array are comparable. The normalized log2-ratios are calculated by subtracting a constant c(A) from the original log2-ratios. This constant is found by estimating a local weighted linear regression and calculating the best-fitting average log2-value from the log2-ratios observed in each data point (219). MA-plot of a normalized sample is shown in Figure 11.

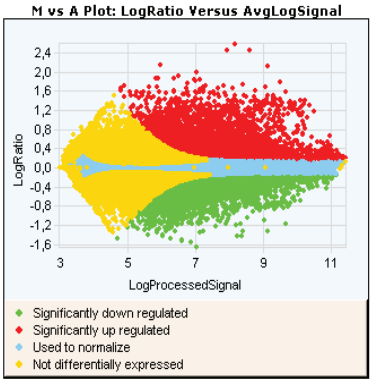


Figure 11 MvsA plot for lowess normalized log2-ratios for MDG011 in this study.

6.5 Mammograms

Mammograms were collected with routine description of the mammograms by the local radiologist. Craniocaudal mammograms were digitized using a high resolution Kodak Lumisys 85 scanner (Kodak, Rochester, New York). We obtained successfully scanned mammograms from 176 women (115 healthy women and 61 breast cancer patients). MD was estimated using the well established, semi-digital University of Southern California Madena assessment method (121). A reader, trained by Giske Ursin, outlined the total breast area using a computerized tool. Giske Ursin then defined the region of interest, being the areas of the breast containing densities except those representing the pectoralis muscle or scanning artifacts. The computer then colors yellow all pixels within the region of interest and above a specified threshold. The colors pixels then represent the area of absolute density. Percent MD is the absolute density area divided by the total breast area (see Figure 12). We use the average percent density for both breasts, whenever an estimate was available from both sides or from the one side we had images if only one side was available. Test-retest reliability was 0.99 for absolute density.

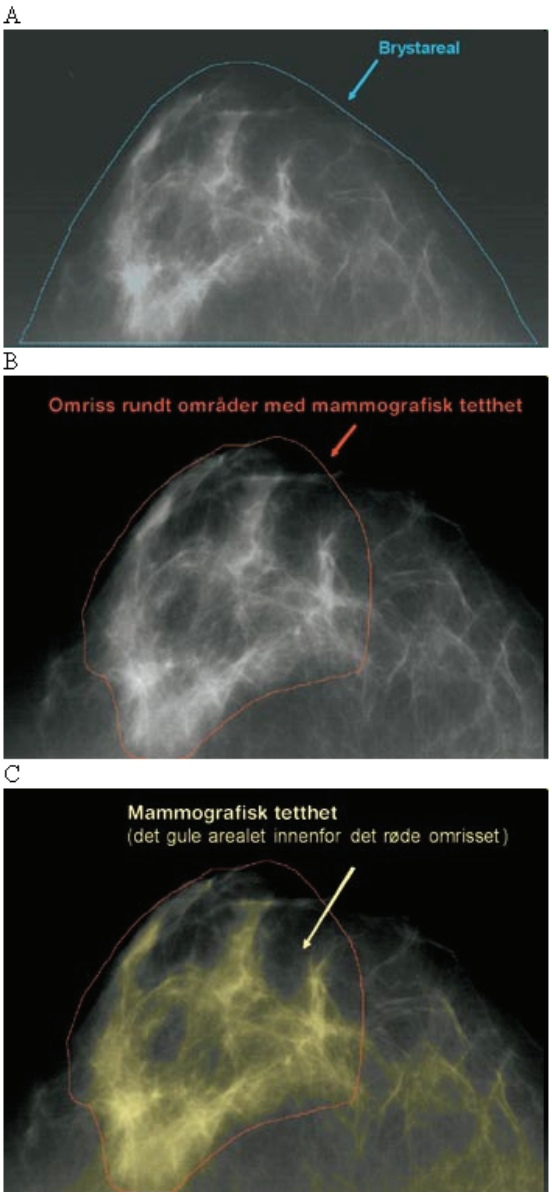


Figure 12 A) The total breast area is delineated. B) The region of interest is defined as the region containing densites, excluding artifacts and the pectoralis muscle. C) The computer colors areas within the region of interest with density above a user-set threshold. Percent area is this dense, colored area divided by the total breast area.

6.6 Exploratory data analysis

Visualization of high dimensional data

Visualization of microarray data in the initial phase may identify outliers and give a broad view of the distribution of the samples included in the study. Principle component analysis (PCA) is a commonly used method of reducing the dimensions of high dimensional data for visualization. The variation in the data is reduced to a few independent components that can be plotted two at a time in a graph. Singular value decomposition (SVD) is a general method for decomposition of a complex matrix into multiple, independent factors. It resembles PCA in that the data can be displayed in a graph representing the contribution of two separate factors at a time in explaining the total variation in the data. In many cases, the two methods will be identical.

Multidimensional scaling (MDS) is a method of visualizing similarities and dissimilarities between objects represented as n-dimensional vectors (n>2). The vectors are mapped to points in a low-dimensional space IRm (where m=2 or 3) suitable for visualization. Points located close to each other represent vectors with a similar shape (egarrays with a similar gene expression pattern).

In this study, the dataset was checked for date- and batch-effect and samples with a questionable quality were re-run. We used MDS and SVD to look for unexpected clustering that could be due to technical issues. By visual inspection, these methods could identify a possible effect of batch, but not of other parameters (egstorage medium), exemplified by multidimensional scaling.

Clustering is another way of visualizing high dimensional data. During this process, the samples are assigned to subgroups/clusters with a similar expression profile. In hierarchical clustering, the output is a hierarchy of clusters depicted in a tree. Hierarchical clustering (Figure 13) is an example of unsupervised learning and can be used for description and visualization of the variation in the data. In agglomerative algorithms, the dendrogram is built from the bottom up, starting with each sample as a separate cluster, joining those with the most similar distribution as the tree grows. In divisive algorithms, the whole set is treated as one cluster splitting it into clusters as the dendrogram builds, but these are more computationally complicated and are rarely used.

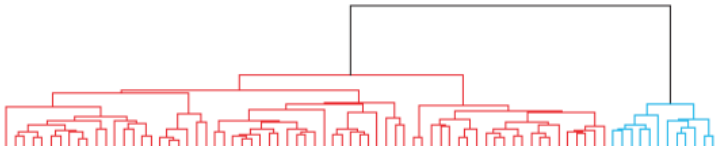


Figure 13 Clustering by agglomerative algorithm where the dendrogram is built from the bottom up.

6.7 Statistical testing

Correcting for multiple testing

A test for statistical significance is a hypothesis testing. The hypothesis we are testing is called null hypothesis and is rejected if our result is less likely to occur by chance, relative to a significance level we have decided. With a significance level of 5%, we say that if our observed outcome is less than 5% likely to occur under the null hypothesis, the null hypothesis is rejected. That means that there is a 5% likelihood of falsely rejecting the null-hypothesis for every test we perform. As we perform an increasing number of tests, the likelihood that one of them will be an unlikely event, even under the null-hypothesis, is growing. To avoid a large number of false positive results, we need to adjust the p-value as we increase the number of tests.

One commonly used method is the Bonferroni-correction. The nominal p-value is multiplied with the number of tests performed. This is a conservative method that is suitable if it is important to control the number of false positives and will typically result in a large number of false negative tests.

False discovery rate (FDR) is a less conservative method used to correct for multiple testing. The FDR is set to the proportion of false positives among all positives that you are willing to accept. Each of the tests performed returns a q-value, which indicates the maximal FDR at which the test becomes significant (221). This is loosely analogous to the p-value and is used in a similar way. FDR is used for correction of multiple testing in our analyses for differentially expressed genes by significance analysis of microarrays (SAM) and for gene ontology analyses by DAVID.

Significance Analysis of Microarrays

SAM identifies gene differentially expressed in two or multiple groups (two-class or multiclass SAM) or according to a continuous variable (quantitative SAM) (222,223). In SAM, each gene is tested for significant difference in expression separately. The test used is a modified t-test. To correct for multiple testing, SAM calculates FDR by permutations. In our study, two-class unpaired SAM was used for analysis of differentially expressed genes between two groups of data. The data were not gene centred for the SAM analysis. A total of 50permutations were used. Quantitative SAM analysis was used to identify genes differentially expressed according to MD as a continuous variable.

Regression models

Regression models can be used to identify the contribution of different independent variables in predicting a dependent variable. Different types of regression models exist which all aim at explaining the variation in a dependent variable Y as a function of one or more independent variables (X1,…,Xp). Linear regression models are structured as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_i X_i + \varepsilon$$

Y is the dependent variable (eg: mammographic density). β_0 is the constant that represents the intercept with the y-axis if the equation is plotted in a two-dimensional graph. β_i is the factor to be multiplied with the explanatory variable X_i and indicates the magnitude and direction of the influence of X_i on the dependent variable. Here, p is the number of explanatory variables included in the model and ε is an error added.

Envisage is a linear model for the analysis of microarray data developed by Sam Robson, University of Warwick (224). The model can be used to perform variable selection but can also be used to check the effect of including variables other than those of interest on the gene expression. The model creates a regression equation for the expression (y) of each of n genes (g_i, i=1-n). The β_i coefficient for each variable indicates how much that variable influences the expression of a gene (y_{gi}). The number of genes whose expression is significantly associated with each covariate is estimated. In this study, Envisage was

used to look for effects of batch, experiment date, storage medium and hospital of inclusion in the gene expression in healthy women. In a model including experiment date, batch, hospital of inclusion and storage medium, experiment date seemed to be of a certain importance of determining the expression (16% significantly changing genes). It is, however a variable that is difficult to use in this model due to the large number of degrees of freedom. In a model excluding experiment date, batch was affecting 29% of the genes. The remaining variables seemed to be correlated with only a small percentage of the genesHospital of inclusion (7%) and storage medium (0%). When the samples are clustered, visual inspection does not identify an effect of these variables. We therefore conclude that there might be an uncorrected effect of experiment date/batch. It does not seem to affect the clustering. We cannot exclude a slight obfuscation of the results.

In order to find a model that suits the distribution of the data, the Akaike information criterion can be used (225). Comparing the Akaike information criterion for different models, the lower criterion indicates the model that best predicts the dependent variable. In this study, we used the Akaike information criterion to select a linear regression model as the model fitting the distribution of the data better than a gamma model. Stepwise variable selection was performed, starting with all variables included in the model. For every step, the variable with the highest p-value was rejected from the model and the model was refitted. This was repeated until all variables included in the model had a p-value <0.05.

Other tests for statistical significance

To test whether the mean of a parameter is different in two groups, a t-test can be used. This test is suitable for continuous variables and for tests between two groups only, and assumes normal distribution within each subgroup. The null hypothesis is that the mean is equal for the two groups. Two-sided t-test accounts for the possibility of either group having largest mean. We have used two-sided t-tests to test different continuous variables (MD, age, BMI) for difference in mean between two clusters. When there are more than two means to be compared (more than two groups/clusters) analysis of variance (ANOVA) was applied. To test for difference between groups/clusters in categorical variables (use of hormone therapy, parity and claudin-low status), the chi-squared ((2)

test was used. When the size of observations for eat least one subcategory was smaller than 5, Fishers exact test was applied (226).

6.8 Bioinformatic Analyses

Gene ontology and KEGG pathway

Gene ontology analysis gives information about biological processes, molecular pathways and cellular compartments overrepresented in a given gene list. The KEGG pathway database has curated lists of genes involved in different pathways. The bioinformatic tool, DAVID Bioinformatics Resources 2008 from the National Institute of Allergy and Infectious Diseases, NIH (227) returns information about gene ontology-terms and KEGG-pathways enriched in the gene list uploaded compared with a general human background. In papers II and III, gene ontology-analysis was performed by the use of DAVID. Functional annotation clustering was applied and the following gene ontology categories were selected: Biological processes (all), molecular function (all) and the KEGG pathway database. We included gene ontology terms with a p-value (FDR-corrected) of <0.01 containing between 5 and 50genes.

UCSC browser

The UCSC browser (228) allows you to browse the genome and zoom in on areas of interest. For all locations in the genome, information about genes, expressed sequence tags (ESTs), microRNA, SNPs and several other trackd can be viewed. In this study, the UCSC browser was used to map the different UGT-probes to the genome and to blast (by use of the BLAT-tool) the probe sequences to verify the homology to the gene the probe was expected to represent.

Gene set enrichment analysis (GSEA)

GSEA is used to test if gene sets (curated or entered by user) are significantly different in two different sets of samples, usually determined by a phenotype of interest (229). The software provided by the Broad Institute (230) rank each gene in the gene set according to the enrichment these genes in each sample set. GSEA was used with user-defined gene sets in paper I.

7. Brief summary of results

Paper I

Gene expression profiles of breast biopsies from healthy women identify a group with claudin-low features

Studies of the normal breast are essential to understand the normal breast biology and to identify biological mechanisms underlying breast cancer risk and initiation. In this paper, we wanted to explore the biology in normal breasts, as representative as possible of the women we meet in a diagnostic setting. The aim was to look for variation and to identify differences in biology that can be used to generate hypotheses about biological risk factors and initiation of breast cancer. Unsupervised hierarchical clustering of whole genome expression microarrays from normal breast tissue from 79 healthy women identified a group of twelve samples that consistently clustered tightly together (cluster 1). The standard epidemiologic data (age, BMI, hormone therapy use) or mammographic density could not explain the difference between these clusters. We did, however, note that none of these twelve samples were referred from the mammographic screening programme, but rather from a doctor due to clinical findings or family history (as opposed to 42% from mammographic screening for the remaining samples). Exploration of the biology of the gene expression in the cluster 1 samples showed that the expression profile resembled that of breast stroma and stem-like cells and shared features with the newly diagnosed claudin-low breast cancer subtype with up-regulation of mesenhcymal genes, down-regulation of cytokeratins and claudins. Biological mechanisms of breast cancer risk and initiation related to these features should be studied in the future. With longer follow-up, we will get true information about breast cancer risk in women belonging to these two clusters.

Paper II

Expression levels of uridine 5'-diphosphoglucuronosyltransferase genes in breast tissue from healthy women are associated with mammographic density

In paper II we identified genes associated with mammographic density in breasts of healthy women. Of 24 genes significantly down-regulated in samples with high MD, three were in the UGT-familiy, coding for enzymes inactivating estradiol by glucuronidation. This down-regulation was seen in women under estrogen-influence; younger women and women on hormone therapy. The UGT-expression in tumor samples resembled the expression in samples from breasts with high mammographic density more than the expression seen in samples from low-dense breasts. *ESR1* was also down-regulated, which may be due to a higher level of estradiol in the breasts with high mammographic density. The hypothesis generated from this study is that a down-regulation of UGT-expression in breasts under estrogen influence increases mammographic density and may also be linked to increased risk for breast cancer. This should be verified in future studies.

Paper III

Serum estradiol levels associated with specific gene expression patterns in normal breast tissue and in breast carcinomas

In paper III we identified genes associated with serum estradiol in normal breast tissue from healthy women and in breast carcinomas. In normal breast tissue, three new genes were differentially expressed according to circulating estradiol (used as a continuous variable) were identified. *SCGB3A1* (*HIN1*) and *TLN2* were up-regulated and *PTGS1* (*COX1*) down-regulated in samples from women with high estradiol levels. *SCGB3A1* is a tumor suppressor, *TLN2* increases cell adhesion which may prevent migration and invasion and *PTGS1* is associated with carcinogenesis. In samples from women with high serum estradiol, the high expression of *SCGB3A1* and *TLN2* alsong with the low expression of *PTGS1* indicates control of cellular growth, proliferation and migration. In tumor tissue, *SCGB3A1* and *TLN2* are down-regulated and *PTGS1* is up-regulated, indicating an increased proliferation and migration.

In breast carcionomas, *AREG* and *GREB1* were significantly up-regulated in samples with higher levels of serum estradiol in quantitative SAM analysis. These genes have previously been found estradiol-responsive in malignant(231) and normal(232) breast tissue. The findings are discussed in relation to previous studies on serum estradiol levels and breast biology.

Paper IV

Identification of SNP markers with putative influence on mammographic density and breast cancer risk

In paper IV, we identified SNPs associated with MD and their association with gene expression and serum estradiol levels. Twenty-eight unique SNPs were found associated with MD in two separate datasets used ($p<0.1$). One of the 28 SNPs, residing in *HSD3D1*, has previously been found associated with MD (233). Of the 28 SNPs associated with MD at $p<0.01$, ten were significant at a standard significance level ($p\leq0.05$). These ten SNPs were residing in *EPOR, UGT2B28, SLC7A5, TBP, PTGER3, SULT2A1* and *UGT2B15* and have not previously been identified. Seven of these ten SNPs associated with MD were correlated with the expression of genes *in cis* of the SNP. Among the genes whose expression was associated with these ten SNPs were *H2AFJ*, one of the genes found associated with MD in paper II. This gene was significant in all stratifications (according to hormone therapy use) in the current study.

Of the 28 SNPs associated with MD at $p<0.01$, two SNPs, residing in *CDK2* and *CYP17A1*, were significantly associated with serum estradiol leves ($p\leq0.05$). The SNP in *CDK2* was also correlated with the expression of two genes (*SCAMP1* and *RPS26*) which were in themselves associated with serum estradiol levels.

8. Discussion

8.1 Sample collection and methodological considerations

"Finding the question is often more important than finding the answer" Tukey, 1980

Study design

Most normal breast tissue biopsies for research purposes are currently taken from histologically normal tissue adjacent to tumor in breast cancer patients or from reduction mammoplasties. Tumor adjacent normal tissue may be influenced by the presence of a tumor in the same organ (234), and mammoplasty reductions tend to be very adipose-rich and may therefore have different expression profiles from the population at risk. In some cases, the source of the normal tissue may bias the results. This present study is a prospective study of normal breast tissue from healthy women. The unique aspect of the study is that the tissue analyzed is from tru-cut biopsies from healthy women who are not subject to any surgery. They are not breast cancer patients with the cancerous effect on the normal tissue and they are not mammoplasty reductions with a possible bias towards an adipose-related biology. That does not, however, mean that there is no bias in our normal samples. These biopsies are taken from women who are referred to a breast diagnostic centre for examination either due to uncertainties in the screening mammogram or due to a clinical finding/risk. This population is likely to have a higher risk of developing breast cancer than the average women in the total population. Hence, the selection of healthy women in this study is biased compared with women in the total population. Our sample set is, however, representative of the population the radiologists meet in the diagnostic setting in a breast diagnostic center.

In the future, representative breast tissue from healthy women may be more easily available. The US National Cancer Institute is now launching a program to collect breast tissue from all stages of breast cancer development, as well as healthy breasts (235).

Another issue is the problem of obtaining a biopsy representative of the breast it is taken from. It is not known how homogenous gene expression is within one breast, and therefore we are currently unable to determine how representative one single biopsy

would be. Further, obtaining a tru-cut biopsy is an invasive procedure and would not be suitable for screening purposes. It could, however, be useful for high-risk groups of women. Most importantly, we lack knowledge of the biology in high-risk breasts and how to identify the earliest stages of breast carcinogenesis. In this study, the radiologists were instructed to take a biopsy from an area of the breast with some density to avoid purely fatty biopsies. This lead to a bias toward inclusion of women with high MD. This is reflected in the fact that, in this study, the mean MD is higher in the healthy women compared with the breast cancer patients. Therefore, direct comparison of the biology underlying MD in the two populations included in the study is not possible.

The women answered questions about parity, height, weight, hormone use and family history of breast cancer. Menopausal status was determined based on serum hormone levels. Women with uncertain biochemical menopausal status were left out from the stratified analyses.

Histopathology of biopsies

The biopsies in this study were taken with a 14 Gauge needle, which allows for large amount of high-quality RNA from tumors, but more limited amounts from normal tissue where the cellular density is lower. Two specific options of histopathologic evaluation of the biopsies from normal breasts were discussed within the project group. Immunohistochemical analysis of the biopsy would require almost the whole biopsy to be cast in paraffin and we would not be able to extract RNA and DNA for the microarray experiments. Imprint was not in routine use in the hospitals and would have made the inclusion procedure more of a burden to the personnel. In addition, the intact adhesion of normal epithelial and stromal cells prevents the cells of normal tissues to shed off the biopsy to the glass slide. Test imprints showed mostly adipose tissue, a couple of stromal cells and no epithelial cells; even if the macroscopic evaluation was that the biopsy was not dominated by adipose tissue. Knowledge about the cellular content of the biopsies would have improved our understanding of the biology greatly, but was, unfortunately not possible to obtain. A third biopsy for histologic examination was refused for ethical reasons. The inclusion was surprisingly smooth and at a later stage, both the health

personnel involved and the ethical committees agreed that we could sample a third biopsy which is now done for our follow-up study.

Whole genome expression profiling

Gene expression is the process in which a gene is transcribed to RNA which subsequently serves as a template for protein production. The pattern of gene expression reflects the biological activity in the cells. We measured gene expression using Agilents 44K whole genome arrays.

The mean RNA amount extracted from normal biopsies was considerably lower than that extracted from tumor biopsies. Correspondingly, more normal samples (n=39) than tumor samples (n=1) were excluded due to low RNA or poor RNA quality. Of the arrays hybridized, three were excluded due to bad technical quality, one tumor sample and two normal samples. The MD of the women whose breast biopsies resulted in successful gene expression profiling (n=79) was significantly higher than the MD of women whose breast biopsies did not yield enough high quality RNA to obtain profiling (n=42) (p=0.02, mean MD=37 versus 29). Low density breasts with higher adipose component are likely to yield less mRNA and this has contributed a certain bias. Both the inclusion criteria and the biological study methods selected for samples with some epithelial and stromal content. The observation that the healthy women had higher MD than the breast cancer patients was true for the samples with successful gene expression profiling, and for the whole cohort of women included and was therefore not only due to technical issues.

Gene expression from small amounts of RNA warrants amplification before hybridization. The amplification step introduces an extra source of error to the experiment. King and colleagues(236) tested the reliability and reproducibility of gene expression experiments using small and larger tissue samples. They found a high correlation between replicates and between small samples (with amplified RNA) and moderate between small and larger tissue samples. They concluded that the biological variability exceeded the technical variability in the gene expression studies (236). They used microdissected epithelial tissue and Affymetrix arrays, but there is no reason to believe that technical reliability and reproducibility should be less using whole tissue and

Agilent arrays. Biological variation between two whole tissue biopsies might off course be greater, due to differences in cellular content of the biopsies.

To get a picture of the overall biology in the normal breast related to MD we decided to extract RNA from the whole biopsy. Our aim was to get an overall picture of the biology at a given point of time and the profiles obtained represent the collective contribution from all cells in the sample. The last decade it has become evident that the carcinogenic process involves not only the epithelial cells, but all its neighboring cells as well (199). Using the whole biopsy for extraction and lacking histopathology, we do not have information about the contribution to the final signature of individual cell types. This will be the focus of future studies.

Mammographic density

Estimation of MD has been done in several different ways. When estimating MD in breast cancer patients, some studies have preferred to use the breast contralateral to the one with cancer for MD estimation (237,238). Estimation of MD in women with no malignancy has followed different reasoning. If there is a lesion in one breast that is diagnosed as benign, one could argue that the estimation should be made on the contralateral breast (analogous to the studies mentioned above) assuming that the lesion might influence the MD estimate. Some prefer to use the ipsilateral breast, arguing that the MD estimation and the biopsy should be from the same breast in order to infer about the biological associations. Bremnes and colleagues (239) preferred to use only the left breast consistently, because the women are healthy and the MD assessment should not be influenced by our more or less well founded suspicions. The same reasoning lies behind the choice of randomly choosing the breast to use for MD estimation as done in Ursin et al (128). Titus-Ernstoff and colleagues estimated MD from both breasts separately, and used the estimation with the breast with highest density (240). Another approach is to estimate MD for both breast and use an average of the two estimated figures. This approach is, perhaps, more likely to embrace the totality of the biology influencing the breast, although one could also argue for using the ipsilateral breast as best representation of the biology in the specific biopsy. A study by Vachon and colleagues concluded that

the risk of breast cancer was predicted equally well regardless of which side was used for density estimation (241).

In the current study, we decided to use an average of the MD estimation from both breasts, whenever available. This was partly in order to encompass the wider aspects of the biology influencing the breasts and partly because we lacked information about which side some of the biopsies were taken from in the healthy women.

Statistical considerations

The gene expression dataset obtained from the microarray experiments of 79 healthy women contained information about 40792 probes, a too high dimension for many of the statistical tools used. A high number of probes included in statistical tests will also dilute the power of the tests due to the problem of multiple testing. Filtering of the gene list is therefore sensible. Using genes with no variation between samples will not help us understand the differences and similarities in the biology of the samples in the study. Therefore gene filtration based on variation (or standard deviation) was performed in adittion to the removal of probes with low quality (a value in less than 80% of the arrays).

Exploratory data analysis differs from confirmatory data analysis in that it generates rather than tests hypotheses. It is used to explore areas with little previous knowledge in order to get an idea about the landscape and use this as a basis for hypothesis testing. The concept of exploratory data analysis was introduced by John W Tukey. In 1980 he wrote "Finding the question is often more important than finding the answer. Exploratory data analysis is an attitude, a flexibility, and a reliance on display, NOT a bundle of techniques" (242).

Exploratory data analysis is typically based on visualization of data. Methods of visualization include commonly used box plots and scatterplots and more complex methods such as multidimensional scaling and unsupervised hierarchical clustering. In paper I, the main aim was an impartial exploration of the data to get a description of the variation and to generate ideas for further studies in order to explore normal breast biology and identify mechanisms contributing to breast cancer risk in healthy women. The most important tool in this paper is therefore hierarchical clustering as an example of unsupervised learning.

The complexity of high-dimension data is too great to grasp without reducing the number of factors. The identification of separate clusters effectively reduces the complexity to more graspable entities. In our case, study of the two sample clusters helped us point to parity and referral source as parameters important in explaining the larger part of the variation. Does this indicate that there is a difference between the clusters in breast cancer risk? If so, what is the mechanism by which the risk increases? The exploration has not given all answers, but shown us enough to enable us to make questions for further analyses. Some of these will be pursued in future projects (se future perspectives).

Many tools exist for clustering and visualization of microarray data, both open source and commercial products. Most researchers find a handful of tools that they like and use. R is an open source language and platform widely used in medical research for bioonformatics. Most new methods are published as R-scripts within a short time. The two main limitations are the need to learn the programming language and the lack of advanced options for visualization and browsing of visual output.

During this study, we developed a tool for exploratory data analysis with the advanced visualization options of the commercial software MatLab (243). Special features needed for publication have been added to the tool as well as statistical methods of determining the number of sample clusters present.

8.2 Biological considerations

"*The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them*." William Lawrence Bragg

Interpretation of gene expression profiles

Identifying gene expression profiles characteristic for subgroups of subjects is one of the most common ways to analyze gene expression data. There is, however, great inconsistency or instability in the profiles generated for the same traits in different studies (244). One study found several different 70-gene signatures predicting breast cancer survival with the same accuracy as the established signature published by vant Veer (245) and there is most often little overlap between the lists (246).

Part of the inconsistency may be explained by use of different platform and technology. In addition, the expression of many different genes is highly correlated and different genes may therefore represent the same process in a signature. Roepman et al showed how this may result in different gene lists with equally good predictive power and an apparent inconsistency (247). In support of this, Yu and colleagues found overrepresentation of the same pathways, represented by different genes, in five separate gene signatures (246).

Another important cause of the inconsistency of different gene lists meant to predict the same trait is, however, that the studies are based on too few samples (245). Ein-Dor and colleagues found that several thousand samples was required to establish robust gene lists for outcome for early breast cancers (248). Increased consistency of the signatures with increasing sample sizes was indeed confirmed by a group that pooled data from different studies and acquired a dataset of a total of 1372 samples (249). Such a pooling is feasible only for traits and sample populations that are extensively studied. As classification of the studied disease improves, the problem of not having large datasets increases. Prediction of risk for each subtype of breast cancer needs inclusion of a large amount of women who develop each subtype and this represents the main challenge in molecular prediction of breast cancer risk. In the near future, increased predictive power may be achieved

through integration of information from different levels, eg micro-RNA, protein expression, SNPs, metabolomics and epigenetics (244).

Variation in normal breast tissue

Whole genome expression profiling of normal breast tissue has previously been performed as a control and reference for tumor tissue. However, during the last decade, there has been increasing interest in the biology in normal breast tissue per se and in relation to breast cancer risk and initiation. One line of interest has been to characterize the gene expression profiles of different cells and tissues co-existing in the breast. Another line of interest is to study gene expression profiles in normal breast tissue in relation to a particular phenotypic trait, such as parity (250), the effect of radiotherapy (56) or MD (55,251). Paper I is pursuing a third approach to the study of normal breast tissue; unsupervised exploration of the variation in gene expression present in the dataset. This exploration revealed that distinct subgroups exist in normal breast tissue. Supervised analyses were included to interpret the initial results as described in the results section.

The biology underlying mammographic density

One study has previously looked at whole genome expression profiles related to MD (55). The published a gene list of 73 genes found differentially expressed between high and low MD. The gene ontology terms associated with the list of differentially expressed genes in Yang et al included tissue morphology and many of the genes were involved in cell-cell signaling. At the pathway level, the analyses pointed toward decreased TGFβ-signaling in breasts with high density. This pathway is important in many processes, including tissue differentiation and apoptosis. There was no overlap between the genes differentially expressed according to MD in Yang et al and in paper II and TGFβ-pathwy genes are not enriched in the genes differentially expressed in paper II. This is probably due to the difference in tissues studied; normal tissue from breast cancer patients as opposed to from healthy women. The relation between the biology in breasts with high and low MD is probably affected by the presence of a tumor. The TGFβ-pathway has a dual role in breast cancer development with a tumor suppressor function in early stages and a promoting role in later stages (252). The alterations in TGFβ-signaling in the tumor

may also affect the surrounding tissue. The relatively high MD in the sample population in paper II may have influenced the interrogation of biological processes related to MD.

MD increases the risk both for ER+ and ER- breast cancer (133). This is different from many of the other risk factors (such as BMI and postmenopausal hormone therapy) which increase the risk of ER+ breast cancer only (253). This suggests a different mechanism for the increased risk of breast cancer from MD. Decreased expression of UGT-genes could be one such mechanism. The function of the UGT-enzymes in inactivating estrogen metabolites (254) suggests that it increases the risk of ER+ tumors only, but this is currently unknown. Our data indicate an association with risk for breast cancer in that the correlation between the UGT2B-probes and MD was low for samples from women who never had breast cancer (-0.01 - -0.36), but high for samples from women with breast cancer (0.3-0.95, particularly for UGT2B15/17 (0.95), UGT2B7 (0.91) and UGT2B17 (0.82). All these cancers were histologically ER+. Further studies are needed to confirm the observed association between UGT-expression and MD and to explore any possible relation to breast cancer risk and development.

High MD may result from a high proportion of cells (epithelial and/or stromal cells) or from high proportions of collagen. There may be different biological processes resulting in biological structures representing density on the mammogram. When percent or absolute density is used, these different mechanisms are not taken into account and information about underlying biological differences may be lost. One way of dissecting the problem of differing types of MD is to use qualitative assessment of the mammograms in addition to the quantitative measurements. The parenchymal pattern of mammographically dense tissue may be classified into glandular/nodular or sheetlike. The nodular pattern may represent a higher proportion of epithelial cells lining the ductal tree, whereas the sheetlike pattern may represent more collagen and possibly other non-glandular stromal structures. The mammograms in this study have been classified according to parenchymal pattern by the epidemiologist, Giske Ursin. Initial analyses identify differential expressed genes between the two growth patterns in dense breasts. This will be analyzed further in the near future (see Future perspectives). Previously, a nodular pattern has been linked with increased risk for breast cancer (255,256). In the

current study, the few women who developed breast cancer after inclusion in the study did not have the same parenchymal patterns.

The genetic component probably account for more than 50% of the variation in MD (145) (146)). Several studies have tried to identify SNPs responsible for this genetic component. Some groups have identified SNPs associated with MD (7,153,156,233), whereas others have found no association (155). A recent study reported that SNPs in the estradiol metabolic pathway was associated with breast cancer, indicating the importance of further studies of the SNPs in this pathway (171). Our study of estrogen-related SNPs associated with MD identified seven new SNPs associated with MD and confirmed one SNP previously reported. This strengthens the hypothesis that the estradiol pathway is important for MD as well as for BC. One major issue of studies of genetic variation is the vast number of SNPs existing in the human genome. Studying all these SNPs simultaneously would result in a low statistical power due to correction for multiple testing. The use of tag SNPs reduces the number of SNPs needed to obtain the biological information somewhat. The number of tag SNPs is, however, vast and would require very large study populations to achieve a reasonable statistical power. Most groups circumvent this problem by selecting a smaller number of SNPs to study. The SNP selection is frequently different for different studies resulting in a small overlap of SNPs from study to study. Few SNPs are therefore verified. Of 281 SNPs included in the current study, we found that five had been studied in relation to MD previously. Only one of these five (rs1047303 residing in *HSD3B1*) had been found significantly associated with MD. We reproduced the previous results in these five SNPs.

Four of the seven SNPs found significantly associated with MD in paper IV were associated with the expression of *H2AFJ*, one of the genes found significantly associated with MD in paper II. These associations may indicate that this gene is mediating a significant proportion of the genetic component of MD. Over the last years, there have been fewer than expected significant SNPs identified in relation to both breast cancer and MD. A verification of the current findings along with an expansion of the SNP-panel studied is warranted to get a complete picture of the biology responsible for the genetic component of MD. Several genome-wide association studies and large epidemiologic studies focusing on SNPs in relation to breast cancer have been launched and results have

started to be published (166,172,173). Some have information about MD (155), but most of these studies do, however, lack breast biopsies for gene expression analysis.

Estrogen-related regulation of breast biology

The three genes whose expression was found associated with serum estradiol levels in paper III (*SCGB3A1*, *PTGS1* and *TLN2*) have all been linked to breast carcinogenesis itself (257) and/or mechanisms possibly important in breast carcinogenesis (258). Much is however unknown about their role in breast biology and breast carcinogenesis. The current study strengthens the need for further exploration of the role of these genes and suggests several studies that will be pursued in the future.

Aromatase (CYP19A1) is responsible for the final conversion of androgens to estrogens in mammary adiopocytes. The expression levels of the enzyme are therefore expected to reflect the level of local production of estradiol. *PTGS1*, in our study found to be down-regulated in breast tissue from women with high serum estradiol, has been found to increase production of prostaglandin E2 which in turn increases the expression of aromatase (CYP19A1) (259) (Figure 15). We did, however, not find a correlation between the expression of *PTGS1* and *CYP19A1* in our material. Nor did we find *CYP19A1*-expression to be correlated to serum estradiol.

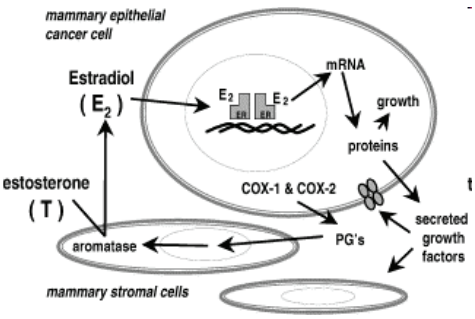


Figure 15 The cyxloocygenases increase prostaglandin E2 activity which induces aromatase and its conversion of androgens to estrogens in adipose cells. Estradiol binds to nuclear ER-receptors that act as transcription factors. From Richards et al (260).

Several UGT2B-genes were found down-regulated in breasts with high MD.These enzymes inactivate estradiol. Samples with high expression of *UGT2B10* tended to have low expression of *CYP19A1* (-0.73 versus -0.54, p=0.09) and come from women with

lower levels of serum estradiol (0.19 versus 0.35, p=0.09). They do, however, have a high expression of *ESR1* (1.95 versus 1.2, p=0.002). The UGT2B-genes do not seem to be up-regulated as a response to high estradiol concentrations in the tissue as might be expected. The anticorrelation of serum estradiol and local expression of *ESR*1 is previously found in breast cancer (231), and in normal breast tissue in monkeys (36) and in mice (261). Does the high level of *ESR1* allow increased impact of the estradiol present and hence reduce the estrogen production (locally and systemically) as well as the inactivation of the hormone? Or is the high expression of *ESR1* a result of the low estradiol available in the breast tissue?

There is considereable uncertainty about the regulation of the expression of these genes and this should be explored in future studies.

In this study, serum sex hormone levels have been studied and several estrogen-related enzymes have revealed significant associations with the study parameters in the various papers. Figure 16 gives a schematic and simpstic overview of human estrogen synthesis and metabolism.

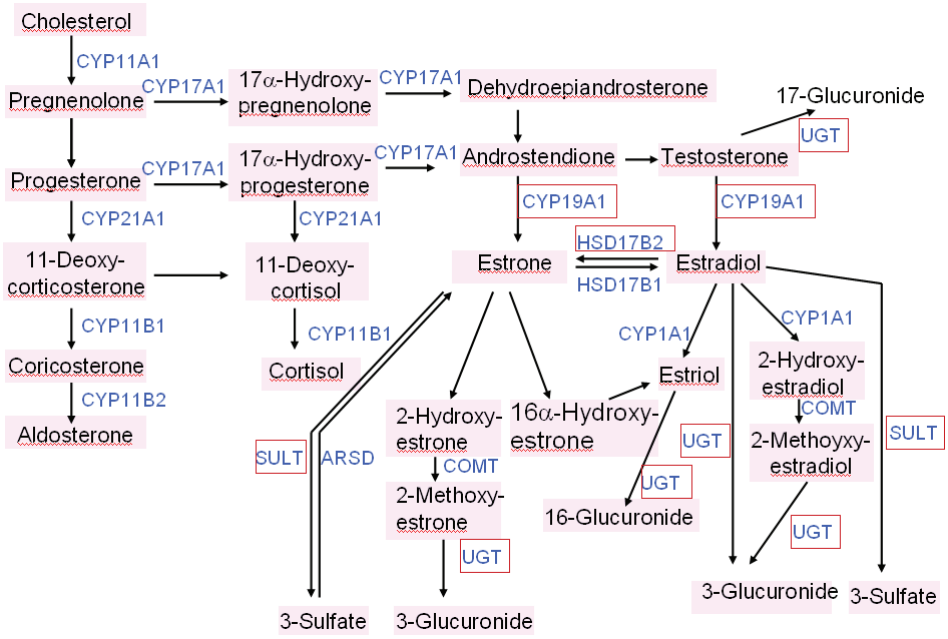


Figure 16 Androstendione and estrogen metabolism adapted form Yoshimura et al (262) and KEGG. The presentation is not complete. Enzymes in blue and hormones/metabolites in black. Enzymes/hormones figuring in the results of the current study are marked in red.

9. Main conclusions and future perspectives

Main conclusions

This study has found that there are distinct subgroups of normal breast tissue and that one subgroup shares features with stromal cells, stem cells and the claudin-low breast cancer subtype.

We have also found that down-regulation of UGT2B-genes in breasts with high MD. Reduced glucuronidation of estrogen and androgen metabolites by UGT2B-genes may increase the stimulation of mammary cells leading to increased MD. This may be through a high number of epithelial or stromal cells or increased production of collagens. Seven SNPs associated with MD have been identified and *H2AFJ* has been put foreward as a putative gene expression mediator of some of the genetic component of MD.

Gene expression of *SCGB3A1* (*HIN1*) and *TLN2* is up-regulated and that of *PTGS1* (*COX1*) is down-regulated in samples from women with high levels of serum estradiol indicating intactregulation of growth control. This regulation is lost in breast cancers where the levels of the tumor suppressor *SCGB3A1* are reduced and the expression of *PTGS1*-expression is increased.

Major unknown issues

This is a small, explorative study and all results need to bee verified in other studies in order to draw final conclusions. The study has, however, also pointed out specific questions that we are unable to answer at this point in time.

1. What are the biological subgroups of high MD and how are they differentially regulated?
2. Is the association of UGT2B-genes with MD restricted to a specific biological mechanism of MD?
3. Is there a difference in risk for breast cancer for women with similar MD with varying local expression of UGT2B-genes?
4. Is the putative role of UGT2B-genes in breast carcinogenesis restricted to ER-positive tumors?

5. Is the cluser 1-phenotype (paper I) specific to the woman or representing traits present in all breasts to a certain degree?

6. Is the cluster 1-phenotype associated with increased risk of breast cancer?

7. Do claudin-low tumors arise from cells with a cluster 1-phenotype?

8. What are the regulatory mechanisms responsible for the cluster 1-phenotype?

9. What are the gene expression profiles associated with high risk for breast cancer?

10. Are there serum markers that may identify high risk for breast cancer or early breast carcinogenesis?

11. Why is SCGB3A1-expression reduced in basal-like tumors compared with other subtypes? Is it due to a myoepithelial cell of origin or transformation to a myoepithelial phenotype?

12. Are the alterations in gene expression according to serum estradiol levels caused by the circulation estradiol or associated due to a confounding factor?

13. Is there a direct regulation of local estradiol-production (measured by *CYP19A1*-expression) by the circulation estradiol?

14. What induces the increase in expression of *PTGS1* seen during carcinogenesis?

15. Is there a relation between parenchymal mammographic patterns and biological features such as:

    a. Expression of specific genes in the breast tissue

    b. Overall gene expression profiles (such as the cluster 1 phenotype)

    c. Serum levels of estradiol

16. ¨How can we combine information from different levels in the cell to characterize normal breast tissue and identify breast cancer risk?

17. Are the SNPs identified in paper IV also associated with breast cancer development?

Future perspectives

Technology capable of genome-wide analyses of human cells and tissue has opened new possibilities for exploration of biological associations. The next few years we will see an integration of genome-wide information from several cellular levels to a wider extent. It has become evident that genomic alterations, methylation and histone acetylation at the DNA-level and expression of mRNA, micro-RNA, non-coding RNA and proteins are linked. The nature of the regulations is currently explored by many. Development of new biostatistical methods may enable genome-wide profiles accounting for alterations at several levels simultaneously.

The main challenges of the molecular sciences today is not obtaining biological information, but rather to interpret the results achieved from the various laboratory analyses. This is primarily a biostatistical challenge, but does also challenge our biological understanding and ability to comprehend complex patterns.

This study has obtained valuable biological material that allows for many future studies. As we have data from DNA and RNA both in breast tissue and blood, integration from different cellular levels would be possible in this material and would provide new information about the biology in normal breast tissue. Analyses of DNA methylation, genomic variation and gene expression in blood are already under way. Exploration of the biology related to different parenchymal mammographic patterns is an obvious line to follow in order to explore the current results. Specifically, genes differentially expressed in dense breasts with differing parenchymal patterns will be identified. The parenchymal patterns will also be explored for differences in other phenotypic traits such as epidemiologic data, serum hormone levels and breast cancer development.

With increasing observation time, the number of women in our cohort of healthy women developing breast cancer is expected to increase. This will provide a unique opportunity to explore the possibility of identifying molecular risk factors and predictors.

This study may be viewed as a pilot study generating hypotheses for future research in new studies. We are currently sampling new biopsies, blood samples, mammograms and questionnaires from a subgroup of the women included in this study. The main aims of this follow-up study is to see how the gene expression pattern in the breast evolve over

time and to find out whether the cluster 1-phenotype (paper I) is specific for each woman or for cellular compartments present in all breasts.

Comparison of gene expression from normal breast tissue from different parts of the same breast will provide knowledge about the variation in gene expression within the breast of each woman. This will be important in the search for local molecular profiles. Such profiles are primarily important in the search for good serum markers. A breast tissue molecular profile with high predictive power may, however, be a clinically alternative for high-risk women.

Other future studies inspired by the questions raised by the current study include the effects of high and low exogenous estradiol administration in the gene expression and protein profiles of normal human cell lines and normal mouse breast tissue. The analysis of further serum proteins such of leptin, adiponectin, IGF1 and IGFBP3 in relation to the current findings is interesting and feasible in a short term perspective.

Breast cancer risk prediction

Breast cancer risk evaluation is only used to a limited extent in Norway today. Genetic counseling is offered to those with known familial clustering of the disease, mainly families with *BRCA1* and *2*-mutations. MD is one of the major risk factors for breast cancer, but is hardly used clinically in Norway. Several risk prediction models are developed and are used to a limited extent in the US. The main information incorporated in these models is family history of breast cancer, epidemiologic parameters and some times MD (175,178-181,263).

At present, mammographic screening every second year is offered to women aged 45/50-69 in Norway. Critique of the one-size fits all approach to screening has emerged with the call for more individualized risk assessment. Better risk prediction could tailor the examinations offered to subgroups of women. The discriminatory power of the risk predicors existing today is, however, limited and not able to identify high- or low risk women with sufficient accuracy. Several alternative strategies for differentiated recommendations according to risk have been suggested (264,265). The idea of assessing an individual breast cancer risk and stratify into screening groups according to this risk is alluring (particularly based on these studies of MD and variation in normal breast tissue).

This could reduce the harm and the cost of screening in the low-risk population and could increase the detection of cancers in the high-risk population. However, predictive tools with higher discriminatory power are needed before such strategies can be applied in large scale.

There are a few examples where differentiated recommendations may be considered based on epidemiologic evidence. Younger women more often develop more aggressive, rapidly growing tumors, leading to a higher risk of interval cancers and late stage cancers supporting annual screening (266). If the screening program is extended to include women below 50 years of age, yearly screening chould be considered for this group. For women older than 50, biennial screening seems sufficient (267). African-American women generally have a lower MD, but a higher risk of developing breast cancer. They also develop triple-negative, rapidly growing, and aggressive tumors more frequently. Earlier entry into the screening program and/or more frequent screening could be considered for this group. New diagnostic methods, such as gamma-imaging, are being studied and may be introduced for improved sensitivity in women with high MD (268).

Breast cancer risk prediction will probably be improved the coming years, with the identification of SNPs, methylation patterns and gene expression patterns in healthy women associated with future breast cancer development. One of the main challenges here is the heterogeneity of the disease combined with the difficulty of obtaining representative normal breast tissue.

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Original papers

Paper I

Gene expression profiles of breast biopsies from healthy women identify a group with claudin-low features

Gene expression profiles of breast biopsies from healthy women identify a group with claudin-low features

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Financial support:
This study was funded primarily by The Research Council of Norway (VDH is on grant no SKO1378) and South-Eastern Norway Regional Health Authority. CMP and MT were supported by NCI RO1-CA138255 and a Breast SPORE grant P50-CA58223.

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Conflict of interest:
The authors claim no conflict of interest.

Running title:
Gene expression profiles in normal breasts

Keywords:
Gene expression, normal breast tissue, hierarchical clustering, claudin-low,

Abstract

Increased understanding of the variability in normal breast biology will enable us to identify mechanisms of breast cancer initiation and the origin of different subtypes, and to better predict breast cancer risk. In this pilot study we have explored the variation in gene expression patterns in breast biopsies from 79 healthy women referred to breast diagnostic centers in Norway.

Unsupervised hierarchical clustering identified 12 samples consistently clustering tightly together (cluster 1) regardless of clustering algorithm and gene filtering used. Genes up-regulated in cluster 1 samples code for proteins involved in extracellular matrix, vascular development, response to hormones and metabolism. Proteins involved in cell-cell junction and plasma membrane were over-represented in the genes down-regulated in cluster 1. Validation in a separate dataset consisting of histologically normal tissue from both breasts harboring breast cancer and from mammoplasty reductions identified a similar cluster with up-regulation of the same functional categories. Comparison of the expression profile of the cluster 1 samples with several published gene lists describing breast cells showed that these samples share characteristics with stromal cells and stem cells, and to a certain degree with mesenchymal cells and myoepithelial cells. The samples in cluster 1 share many features with the newly identified claudin-low breast cancer intrinsic subtype, which also shows characteristics of stromal and stem cells.

Further studies are needed to fully elucidate the variation in the biology of normal breasts, its relation to breast cancer risk and possible link to the origin of the different molecular subtypes of breast cancer.

Introduction

Early diagnosis of breast cancer is essential for reducing both mortality and morbidity of the disease. Knowledge of the initial steps of breast carcinogenesis is important for development of early detection strategies. Breast carcinogenesis, with the transition of normal breast epithelial cells through hyperplasia to invasive cancer, is increasingly well understood (1,2), but there is uncertainty as to the exact mechanisms of tumour initiation and in which cells these first steps occur (3). In order to obtain a better understanding of breast cancer biology, breast carcinogenesis and origin of the different molecular subtypes of breast cancer, information about normal breast biology and its variability among women is essential.

In breast carcinomas, the variability of gene expression is extensively studied. Several expression subtypes have been identified (4,5). These subtypes are partly believed to originate from different cell types of the breast, the luminal subtypes from luminal epithelial cells and the basal-like subtype from a myoepithelial or a possible luminal progenitor cell type (6). Recently, an additional subtype has been identified (7), the claudin-low subtype, which, based on gene its expression profile, is characterized by low expression of luminal markers and high expression of mesenchymal markers. This subtype is associated with bad prognosis and is thought to be derived from stem cells (8).

The normal breast consists of epithelial cells, extracellular matrix with stromal cells, adipose tissue and breast stem cells that reside in the stem cell niche (9). The stem cell niche prevents the epithelial stem cells from differentiating and is defined by stroma (10). Epithelial breast cells may be of luminal or myoepithelial type and they may undergo epithelial-mesenchymal transition and gain mesenchymal characteristics. Several groups have published lists of genes characterising these various cell-types (11-19).

Whole genome expression profiling of normal breast tissue (all cell types included) from women with no malignant disease has been performed to a limited extent in studies with other aims and with few samples included (5,20-22). In this study we explore the expression profiles of normal breast tissue from a series of healthy women in order to characterize the variability that exists and associate that to demographic data like age, body mass index, hormone therapy use and parity to improve our understanding of normal breast biology. The expression profiles obtained mirror the combined gene activity of the different cell types in the biopsy, reflecting a fingerprint of the breast tissue of that particular woman. Analyzing normal breast tissue may identify biological significant subtypes of normal breast tissue from healthy women. This could be of importance for understanding the different expression patterns seen in the various breast cancer subclasses (4,5).

Materials and methods

Materials

MDG – mammographic density and genetics

The mammographic density and genetics (MDG) project was initiated to study the breast biology of healthy women and in particular the biological/genetic basis for mammographic density. Women included in the study were recruited from several mammographic centers in Norway between 2002 and 2007 as previously described (23). Most women were referred to the mammographic center after some irregular or questionable findings in an initial mammogram. A total of 120 women who were evaluated as cancer-free from routine diagnostic procedures were included in the study. Women with some visible areas of mammographic density were included in order to obtain biopsies from these areas with epithelial and stromal components. If there was a suspicious lesion in one breast, the study biopsy was taken from the breast contralateral to the lesion. Women who used anticoagulants, had breast implants, were pregnant or breast feeding were excluded. All women signed an informed consent and answered a questionnaire with information about parity, family history of breast cancer and hormone use. Breast biopsies and blood samples were collected. The hospital research protocol board and the regional ethical committee (ref: S-02036) approved the study. Data from the questionnaires was stored in a database organized by the Office for Clinical Research at the Oslo University Hospital; Radiumhospitalet.

Core biopsies were taken for RNA-extraction using a 14 gauge needle. The biopsies were taken from an area without pathology but with some mammographic density. Six healthy women included by one hospital, had the biopsy taken from a non-malignant lesion (five from fibroadenomas and one from a microcalcification). The biopsies from one hospital (77 women) were fresh frozen and stored at -80°C. The remaining hospitals placed the biopsies directly on RNAlater (Applied Biosystems/Ambion, Austin, TX) before transportation and storage at -80°C.

Mammographic density was estimated using the University of Southern California Madena assessment method (24) as previously described (23). Briefly, the total breast area was outlined by an operator. The area containing densities and excluding the pectoralis muscle and artifacts was marked and the threshold set to select the densities within this area. Percent density is the dense area divided by the total breast area and was used as a measure of mammographic density. Information about which of the included subjects that had developed breast cancer by April 2010 was collected from the Norwegian Cancer Registry.

Other datasets

Gene expression profiles from breast biopsies of healthy women included in the MDG study were compared with one published and two unpublished gene expression datasets. The two unpublished dataset were from the AHUS hospital. The AHUS1-samples were histologically normal tissue collected from two different cohorts; breasts harboring breast cancer (hereafter called cancer normals) and mammoplasty reductions. The AHUS2-

samples were collected from different sources selected to different proportion of fatty and connective tissues. Breast tissue was sampled from mammoplasty reductions, fibroadenomas and normal tissue from breast cancer mastectomies. In addition, subcutaneous fat was collected from the abdominal area. The samples were grouped into biopsies with high and low fraction of fat tissue based on visual inspection. In both AHUS datasets, RNA was extracted from whole tissue. In addition, one published dataset containing reduction mammoplasties and cancer normals was used (20).

Previously published lists of genes differentially expressed between epithelial cells and stem-like/progenitor cells, stromal cells, myoepithelial cells or epithelial cells after epithelial-mesenchymal transition were used to describe our dataset. The genes from each publication are listed in Supplementary file 1.

Gene expression analysis

RNA-extraction and hybridization to microarrays were done as previously described (23). Briefly, RNeasy Mini Protocol (Qiagen, Valencia, CA) was used for RNA-extraction. Agilent Low RNA input Fluorescent Linear Amplification Kit Protocol was used for cDNA-synthesis, transcription and labeling of RNA with cyanine 5 (Amersham Biosciences, Little Chalfont, England) for the samples and cyanine 3 (Amersham Biosciences, Little Chalfont, England) for the Universal Human total RNA reference (Stratagene, La Jolla, CA). After exclusion of 38 samples due to low amount of RNA or poor RNA-quality, 82 samples were hybridized onto two-channel 44K Agilent Human Whole Genome Oligo Microarrays (G4110A) (Agilent Technologies, Santa Clara, CA). Three arrays were excluded due to poor quality, and 79 samples were included in further analyses and are available in Gene Expression Omnibus (GEO) GSE18672.

Data processing

An Agilent scanner (Agilent Technologies, Santa Clara, CA) was used for scanning and Feature Extraction 9.1.3.1 (Agilent Technologies, Santa Clara, CA) was used for data processing. Normalization was done by locally weighted scatterplot smoothing (lowess) and flagged spots were removed. The Stanford Microarray Database (SMD) (http://genome-www5.stanford.edu//, 8/31/2010) was used for data storage. For further analysis the log 2 transformed data were used. The genes were filtered so that only genes with 80% good data and a log2-value of more than 1.6 standard deviation away from the mean in three samples or more were included leaving 9767 probes. The data were gene-centered for cluster analysis, but not for other analyses. Missing values were imputed in R using the method impute.knn in the library impute (http://rss.acs.unt.edu/Rdoc/library/impute/html/impute.knn.html, 8/31/2010). The AHUS1-dataset was filtered to include the probes in the filtered MDG-dataset, leaving 8519 probes.

The data were checked for effect of handling (fresh frozen versus RNAlater) and batch using significance testing, Envisage (http://www2.warwick.ac.uk/fac/sci/moac/currentstudents/2003/sam_robson/linear_models/, 8/31/2010) and visualization by multidimensional scaling and single value decomposition. Samples with questionable array quality were re-run. The conclusion was

that a slight effect of batch and date of hybridization using uncorrected data is seen, but this did not affect the clustering. Fisher exact and chi-squared tests were used to analyze for difference between our two main clusters using batch, experiment date, storage medium, RNA-concentration and hospital of inclusion as variables. The results of these were all negative showing no effect of sample handling or collection site. Also, there was no correlation between sampling method/storage medium and RNA-amount or quality.

Statistical Analysis

Clustering was performed in MatLab (version R2007b) (The MathWorks Inc., Natick, MA) using ward linkage and Euclidean distance measure. The gap statistic was used to determine the number of clusters (25). Two-sided t-tests (assuming equal variance) and chi-squared/Fisher's exact tests were used to test for significance of different phenotypic variables between the different clusters. Significance Analysis of Microarrays (SAM) (version 3.02, http://www-stat.stanford.edu/~tibs/SAM/, 8/31/2010) (26) for Excel with 500 permutations was used for analysis of differentially expressed genes. The empirical null distribution was estimated to ensure that the genes identified as differentially expressed between the two clusters were not merely the tails of a wider null distribution. Unsupervised hierarchical clustering was performed using the complete gene list filtered as described above. Supervised analyses were performed using different published gene lists to look for similarities with the different cell types from which the respective gene sets were derived.

Prediction of the claudin-low subtype was done using the claudin-low predictor developed in Prat et al (27). An expression dataset with 807 genes and 52 cell line samples (described in Neve et al, (28)), of which 9 were classified as claudin-low, was merged with our data using Distance Weighted Discrimination (29) with the 52-sample dataset used as the training data. In the same software, the single sample prediction (SSP) function with Euclidean distance was applied on the adjusted datasets and then used to define claudin-low samples in the test set.

A similar predictor was developed for prediction of the previously identified intrinsic subtypes. A dataset containing both the original intrinsic subtypes and the claudin-low subtype (7) was merged with our data set as described above for the cell line data. The Herschkowitz-dataset was used as the training data. The single sample prediction was applied to assign expression subtypes to the samples.

Microsoft Access 2003 was used to limit our dataset to the gene lists of interest. Hierarchical clustering was performed to see whether the gene list of interest separated the cluster 1-samples from the remaining samples in our dataset. SAM of cluster 1 versus cluster 2 was performed to identify genes from the published gene list that were differentially expressed between cluster 1 and cluster 2. Tests for significance between the number of up- and down-regulated genes (false discovery rate (FDR)<10%) between the two clusters identified and the cell types in question were performed. Gene set enrichment analysis (GSEA) (version 2) (http://www.broadinstitute.org/gsea/, 8/31/2010) with 1000 permutations was used to check for significance of the gene lists in separating the clusters. DAVID 6.7 (http://david.abcc.ncifcrf.gov/home.jsp, 8/31/2010) was used to

identify gene ontology terms and KEGG pathways significantly enriched in the lists of genes differentially expressed between the two main clusters with an FDR<0.01 considered significant.

Clustering combining the MDG dataset with datasets containing biopsies from normal tissue containing different proportions of adipose tissue was performed to see whether samples in cluster 1 consistently clustered with samples with a high fraction of fat tissue and was driven by a high number of adipocytes.

Results

Unsupervised hierarchical clustering

Unsupervised hierarchical clustering of the expression of 9767 genes in the 79 breast biopsies separated the samples into two main groups, confirmed by the gap statistic (25)(Figure 1). The smaller cluster (cluster 1, far right), consisting of twelve samples, consistently clustered tightly together regardless of clustering method and gene filtering used. There was a significantly higher proportion of women referred to mammography due to increased risk; family history of breast cancer (4) or a palpable breast lump (5) in cluster 1 compared to the remaining women (cluster 2) although no malignancy was found in any of the women included in the study by standard diagnostic procedures. There was a borderline significance that more women belonging to cluster 1 were nulliparous, compared to cluster 2. There was no difference in age, age at first birth, hormone use, body mass index or percent mammographic density between women belonging to the two clusters (Table 1).

Differentially expressed genes

SAM revealed 2621 genes differentially expressed between cluster 1 and cluster 2 with an FDR=0, of which 1516 were up-regulated in cluster 1 (Supplementary file 2).

Genes up-regulated in cluster 1 were enriched for the gene ontology terms extracellular region, vascular development, response to hormone/insulin stimulus, glucose and triclyceride metabolism, plasma membrane, cell motion, protein dimerization, regulation of inflammatory response and mitochondrion. Genes down-regulated in cluster 1 were enriched for the terms of proteins involved in actin-binding, adherens junction, cytoskeleton and the plasma membrane (Supplementary file 3, Table S1). Gene ontology terms associated with subsets of genes in the various gene clusters (A-E) are shown in Figure 2.

Supervised analyses

In order to explore the nature of the cells in the biopsies of cluster 1, we used previously published gene lists describing stroma (17,18), breast stem cells (15,19,30), myoepithelial cells (12,14), progenitor cells (14), mesenchymal cells (13), high-risk normal cells (16), epithelial cells from parous women (31), intrinsic genelist (5) and a genelist for prediction of the claudin-low subtype (27).

Both hierarchical clustering, SAM analysis and GSEA indicated that the expression in the cluster 1-biopsies resembled expression in stem-like cells and stromal cells (Table 2) (Supplementary file 3, Table S2). There were also certain shared expression characteristics with progenitor cells, mesenchymal cells and myoepithelial cells. More detailed information about the cells used when generating the gene lists, the samples used and the number of genes from the respective gene lists differentially expressed in our clusters are listed in Supplementary file 3, Table S3. The cluster 1 samples were not

associated with the expression profiles of any of the original breast cancer subtypes (5). However, when a gene list developed to classify the newly identified claudin-low subtype was used (27), we found that the cluster 1 samples were highly associated with the claudin-low gene expression profile (Table 2). This was confirmed when we used this method to create a predictor for one subtype at a time. All samples in cluster 1 were classified as claudin-low as opposed to only three samples from cluster 2 (Figure 1) and the cluster 1 samples were not assigned to any of the other subtypes tested using these predictors. In figure 2, selected genes associated with the claudin-low subtype, stem cells, mesenchymal cells, stroma and epithelial cells and their expression in cluster 1 are shown. Hierarchical clustering based on the various gene lists is shown in Supplementary file 3, Figure S1. These analyses could not confirm any association of cluster 1 with parity (31).

When the filtered expression dataset was clustered with three separate datasets including biopsies from breasts of healthy women with high and low content of fatty tissue (two unpublished and one published (20) dataset), the samples did not cluster according to fat-content (Supplementary file 3, Figure S2).

Four of the women from the cohort have been registered with a breast cancer diagnosis, one before she was included and four had developed the disease after inclusion, all in the breast contralateral to the biopsy. The samples from these five women did all belong to cluster 2. The observation time varied from 34 to 86 months with a mean of 59.1 and a median of 58. All five cancers were estrogen receptor positive. Two of the breast cancers developed after inclusion were infiltrating lobular carcinoma, the other three cancers had ductal histology.

Validation

Unsupervised hierarchical clustering of the AHUS1-dataset yielded two distinct clusters of samples (Figure 3). The smaller cluster (n= 18) includes 8 reduction mammoplasties, while the larger cluster (n=22) included 6. A total of 3102 of 8519 probes were differentially expressed between the two clusters using SAM. Of the 2045 genes up-regulated in the smaller cluster with an FDR of 2%, 1057 were also up-regulated in cluster 1 in the MDG dataset whereas none were down-regulated. Of the 2278 genes down-regulated in the smaller cluster with and FDR of 2%, 962 were down-regulated in cluster 1 and none were up-regulated.

Gene ontology terms enriched in the genes up- regulated in the smaller AHUS1-cluster was very similar to those found in the cluster 1 samples, including: response to hormone/insulin, glucose and triglyceride metabolism, vasculature development, mitochondrion, membrane, protein dimerization and regulation of inflammatory response. In addition, the gene ontology terms vitamin B6-binding , iron ion binding and aerobic respiration were enriched in the genes up-regulated in the smaller cluster of AHUS1. The genes down-regulated in this smaller cluster were enriched for adherens junction, acting binding, extracellular matrix and cytoskeleton – the majority of terms

being in common with those down-regulated in the cluster 1 samples. The full list of gene ontology terms for both datasets is available in Supplementary file 3, Table S1.

Application of the claudin-low predictor on the AHUS1-samples showed that no samples in the larger cluster were assigned claudin low as opposed to 14 of 15 in the smaller cluster.

Discussion

Little is known about gene expression patterns in normal breasts. We have identified a cluster of twelve normal breast tissue samples (cluster 1) that cluster tightly together using different clustering algorithms and different gene lists and that share characteristics of stromal cells, stem cells and the claudin-low phenotype.

The cluster 1 samples have a reduced expression of the epithelial defining keratin genes and have an up-regulation of several mesenchymal markers such as *TWIST1*, *SPARC* and *VIM*. This may lead to the hypothesis that the cluster 1 samples represent more immature or dedifferentiated epithelial cells, and/or enrichment for stromal cells. This is supported by our findings that the cluster 1 samples have an expression of genes that resembles published gene lists characterizing stromal tissue and have an overrepresentation of gene ontology terms associated with the extracellular matrix. Reliable and specific stem cell markers are still unavailable (32), but cells in the cluster 1 samples show similarities with stem-like or progenitor-like cells.

The interindividual differences observed may reflect true differences between women with different risk or exposure histories, or may represent different normal tissue subtypes that are present within a single woman, at different sites in the breast, at different times during the lifespan or in different proportions. For example, stem cell niches may be oversampled in the cluster 1 biopsies.

Stem cell niches are thought to be present in the breasts of all women, but some women may have more than others. The immature breasts of nulliparous women may contain larger volumes of stem cell niches than the post-lactationally involuted breasts. This could explain why there are more nulliparous women in cluster 1 than in cluster 2. Understanding the intra- and inter-individual variation in normal breast tissue is important and this investigation raises the question as to whether the clustering patterns observed represent only a fraction of women or if all women have cells/niches with these characteristics, with some women having a higher fraction than others.

The stem cell niche refers to a zone of the breast epithelium where stem and progenitor cells reside. The microenvironment constitutes the niche and influences the stem cells (33)(for review, see (34)). This would explain the combined stem-like and stromal-like characteristics identified in cluster 1 samples. In breast cancer, the stem cell niche may contain mesenchymal cells derived from the normal breast stroma or recruited from the bone marrow (10) and the current results raise the hypothesis that mesenchymal cells may be present in normal breast stem cell-niches. The link between mesenchymal and stem cell traits is also made clear by Mani and colleagues who showed that immortalized breast cells undergoing epithelial-mesenchymal transition acquire stem-cell like characteristics and that normal mouse mammary stem cells express mesenchymal markers(35).

This study is not designed to predict risk of developing breast cancer. However, when we apply the Chen risk predictor (16), the cluster 1 samples tend to have a slightly decreased risk. There is no difference in mammographic density, one of the strongest risk factors for

breast cancer, between the two clusters (Table 1). The malignancy risk predictor is dominated by proliferative genes. The fact that the cluster 1 samples are associated with a low malignancy risk profile may be a reflection of the low expression of proliferative genes. Low proliferation rate is also seen in stem cells. Looking at the referral source, women in cluster 1 are mainly referred to the mammographic centers due to palpable breast lumps or positive family history and not from the screening program. There were, however, no breast cancers in cluster 1 as opposed to one previous and four developed breast cancers in cluster 2. This is not statistically significant, and we cannot conclude about the breast cancer risk in women belonging to these two clusters. It is, however, worth noting that all the cancers did arise in the breast contralateral to the biopsy. That may be explained by the fact that, if there was a lesion present in one of the breasts at the mammogram, the biopsies were taken from the breast contralateral to the lesion. All the breast cancers were estrogen receptor positive. Since the cluster 1 samples have a stem-like gene expression profile and have certain myoepithelial/basal characteristics, one may speculate that these women, if they develop breast cancer, will have a greater proportion of estrogen receptor negative cancers.

All the 12 samples in cluster 1 were classified as claudin-low, compared to only three of the remaining 67 samples. Similarly in the validation dataset, the claudin-low samples were exclusively in the smaller cluster. The claudin-low subtype is developed for classification of breast cancers and was not thought to be a group of normal breast samples. The claudin-low nature of the cluster 1 samples is, however, striking. Down-regulation of E-cadherin, occludin, claudin 3, 4, 7 as well as up-regulation of the mesenchymal genes and SNAI2 is in line with the features described in claudin-low tumor samples. The low expression of ESR1 corresponds with the estrogen receptor negative trend of the claudin-low subtype (7). The claudin-low tumours are thought to arise from mammary stem cells (8). The hypothesis that the cluster 1-samples are enriched for immature cells is further supported by the down-regulation of GATA3 seen in these samples compared to the cluster 2 samples ($p=3.8E-9$), a protein that is also down-regulated in claudin-low samples (27).

The biopsies used in this study are unique in that they represent the group of women that are examined at breast diagnostic centers. Since the sample size is small, the use of additional datasets may indicate if the current results represent a larger population. The AHUS1 dataset consists of two main types of samples; mammoplasty reductions and cancer normals. Mammoplasty reductions are widely used as representing normal breast tissue, although one can expect the biology to be slightly biased toward fat-related processes. Cancer normals may be influenced by the biology in the tumor (36) or they may represent normal tissue in high-risk breasts (37). A dataset consisting of these two tissue-types, therefore represent a variety of normal tissue, despite its' obvious shortcomings. The fact that the AHUS1 dataset clusters into two clusters with biology similar to those seen in the MDG dataset is interesting and indicates that these results may be reproducible in similar populations.

The reduced expression of epithelial surface makers may be explained by a large component of adipocytes in the biopsies. This is, however, unlikely, as the biopsies were

taken from mammographic dense areas. In addition, when this dataset was clustered with other datasets containing biopsies from normal breast tissue with varying proportions of fatty tissue, the cluster 1 samples do not segregate with the adipocyte-rich biopsies (Supplementary file 3, Figure S2).

There was a greater proportion of nulliparous women in cluster 1. The association between cluster and parity was, however, not confirmed using a gene list describing post-pregnant epithelial cells (31)(Table 2 and Supplementary file 3, Table S2 and Figure S1). The breasts of nulliparous breasts are not fully matured and the fraction of differentiated epithelial cells is lower than in post-pregnant breasts. The genelist published by Asztalos et al is short and may not capture all parity-related gene expression alterations. The cluster 1 samples may represent more immature breasts with increased number of type 1 lobules, but this study does not give enough power to conclude, and the association between the cluster 1 type gene expression profile and parity needs to be elucidated further.

The difference between cluster 1 samples and the remaining normal samples could be due to difference in fractions of the cell types present in the biopsies. For ethical reasons, the number of biopsies pr woman was limited and we did not have enough tissue to do both RNA-extraction and get histology. The lack of histology of the biopsies prior to extraction prevents exact knowledge of the cell types contributing to the expression profiles. It has become evident that the development and progression of breast cancers are not limited to epithelial cells and that the total microenvironment is important. Approximately 95% of normal breast tissue may be composed of stroma, and therefore cell type differences in stroma are most likely captured rather than subtle differences in epithelial content. For evaluation of the putative interplay between all the cells at this location of the breast, expression analysis of the entire biopsy provides the most realistic picture of the situation. Previous studies have shown that different biopsies from whole one tumor share similar gene expression profile (4). The variability of gene expression from different locations of one breast is not known, but King and colleagues have shown that microdissected and bulk tissue samples from normal breasts have a high similarity in gene expression and that such technical differences are minor compared with biological differences (38).

This study is limited by the relatively low number of women included. Larger datasets with several biopsies representing different parts of the breast will be needed to allow further elaboration of the variation in the normal biology of the breast.

Conclusion

Gene expression analyses of biopsies from breasts of healthy women show two main groups of expression patterns. The samples of the smaller group of biopsies cluster tightly together independent of clustering algorithm and gene filtering used. These samples share characteristics with stromal cells and stem cells and are all classified as claudin-low. These findings are validated in a separate dataset of normal breast tissue. Whether these characteristics represent traits of the woman or cell niches present in all breasts is unknown. This cluster may represent the stem cell niche, defined by stromal tissue and containing stem-like cells. There are more nulliparous women in this cluster. The described signature may be a feature more prominent of the immature breasts of nulliparous women. We cannot conclude about breast cancer risk between the two clusters, although we see an overrepresentation of women a positive family history of breast cancer or a palpable breast lump in the smaller cluster. Further studies are needed to verify the hypotheses generated by this pilot study.

Acknowledgments

We thank all the women who participated in the study and all the personnel in the hospitals who made the inclusion of these women possible, in particular the responsible radiologists: Einar Vigeland, Rolf O Næss and Else Berit Velken. We would also like to thank Lars Ottestad for help in the initiation of the project and Hilde Johnsen and Caroline Jevanord Frøyland for lab assistance.

Abbreviations

SAM: significance analysis of microarrays, FDR: false discovery rate. MDG: Mammographic density and genetics.

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Table 1 Women included in the study, descriptive statistics

		all non-cancer (%)	cluster 1 (%)	cluster 2 (%)	p-value (cluster 1 vs 2)
Age	mean	50.2	49.3	50.4	0.52 *)
	<50	31 (39)	5 (42)	26 (39)	
	50-69	45 (57)	7 (58)	38 (57)	
	missing	3 (4)	0 (0)	3 (4)	
Parity	0	10 (13)	4 (33)	6 (9)	**0.05** †)
	1+	65 (82)	8 (67)	57 (85)	
	missing	4 (5)	0 (0)	4 (6)	
Age at first	mean	24.4	24	24.4	0.21 *)
birth	no children	10 (13)	4 (33)	6 (9)	
	<25	30 (38)	4 (33)	26 (39)	
	25+	26 (33)	3 (25)	23 (34)	
	missing	13 (16)	1 (8)	12 (18)	
Hormone	never	55 (70)	8 (67)	47 (70)	0.62 †)
therapy	current	11 (14)	2 (17)	9 (13)	
use	past	6 (8)	0 (0)	6 (9)	
	missing	7 (9)	2 (17)	5 (7)	
Body	mean	24	23	24	0.24 *)
mass	<20	5 (6)	2 (17)	3 (4)	
index	20-<25	44 (56)	6 (50)	38 (57)	
	25-<30	21 (27)	4 (33)	17 (25)	
	30+	6 (8)	0 (0)	6 (9)	
	missing	3 (4)	0 (0)	3 (4)	
Mammographic	mean	37	40	37	0.62 *)
density	0-<23	19 (24)	2 (17)	17 (25)	
	23-<37	21 (27)	4 (33)	17 (25)	
	37-<52	19 ((24)	3 (25)	16 (24)	
	52+	17 (22)	3 (25)	14 (21)	
	missing	3 (4)	0 (0)	3 (4)	
Serum	mean	0.27	0.30	0.27	0.81*)
estradiol	0-<0.1	27 (34)	3 (25)	24 (36)	
	0.1-<0.2	20 (25)	4 (33)	16 (24)	
	0.2+	31 (39)	5 (42)	26 (39)	
	missing	1 (1)	0	1 (1)	
Referral	risk/lump	32 (41)	9(75)	23 (33)	**0.002** †)
source	screening	28 (36)	0 (0)	28 (42)	
	unknown	19 (24)	3 (25)	16 (24)	

*) Two-sided t-test for continuous variables

†) Fisher exact test for categorical variables with <5 observations in certain cells

Table 2 Comparison of cluster 1 and cell types/subtypes from published gene lists. Chi-squared test is used to illustrate the extent to which genes describing different cell types are equally regulated in the two clusters. Significant p-values are in bold type. For more information on the publications and comparisons, see Supplemental file 1, Table S3.

Comparison	Reference	Cluster 1 resembles	p-value
Epithelial vs stem-like cell	Shipitsin, 2007	Stem-like cell	**2.20E-16**
Stroma vs epithelium	Finak, 2006	Stroma	**2.20E-16**
Mesenchymal vs epithelial	Jechlinger, 2003	Mesen-chymal	**6.90E-14**
Revised subtypes	Herschkowitz, 2007	Claudin-low	**1.52E-12**
Fibroblasts vs epithelial cells	Casey, 2008	Fibroblasts	**1.9E-12**
Risk predictor	Chen, 2009	Low risk	**1.30E-09**
Stem-like cell vs epithelial	Liu, 2007	Stem-like	**1.30E-05**
Myoepithelial vs progenitor	Raouf, 2008	Progenitor	**2.40E-05**
Luminal vs progenitor	Raouf, 2008	Progenitor	**0.001**
Stem-like vs progenitor cells	Villadsen, 2007	Lineage restricted progenitor	**0.008**
Myoepithelial vs luminal	Jones, 2004	(Myepithelial)	0.06
Classical subtypes	Sorlie, 2001	-	0.76

*) Chi squared test for significance

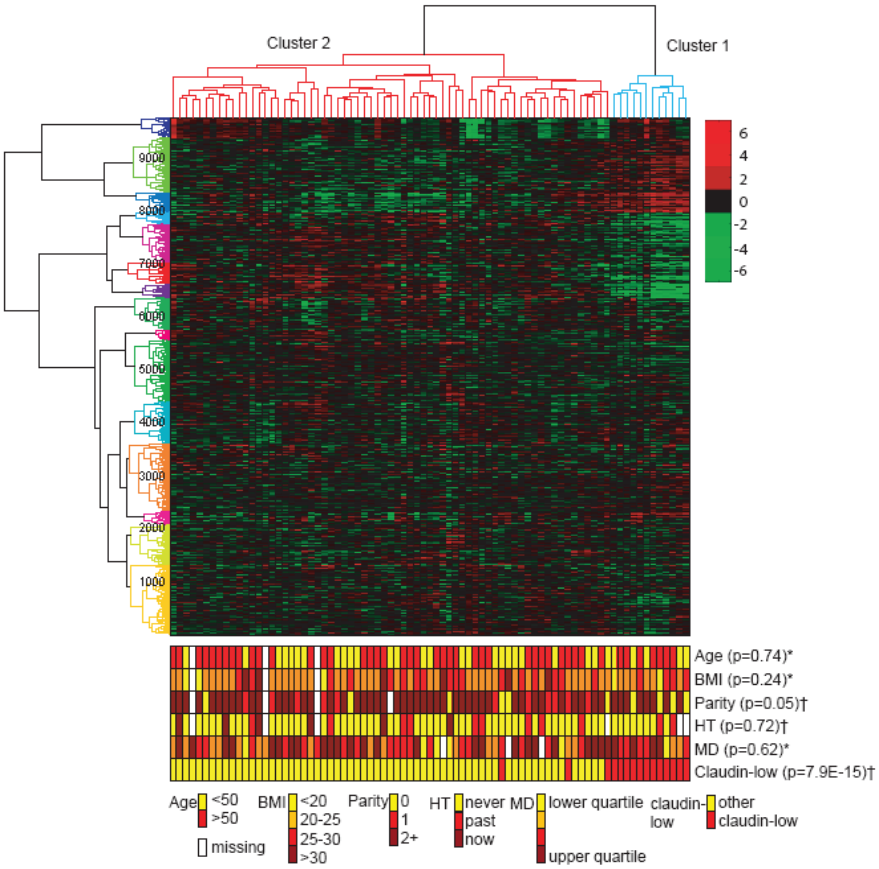


Figure 1: Unsupervised hierarchical clustering of 79 samples from healthy individuals and 9767 genes filtered on variation. Phenotypes with tests for significant difference in values between cluster 1 (blue) and cluster 2 (red). Continuous variables are categorized for the illustration, but significance tested as continuous variables. P-values from two-sided t-tests assuming equal variance for continuous variables (*) and chi-squared tests (†) for categorical variables are given. The numbers along the y-axis denotes the number of genes. Age= Age at time of inclusion. BMI: Body mass index. HT: Use of hormone therapy. MD: Mammographic density.

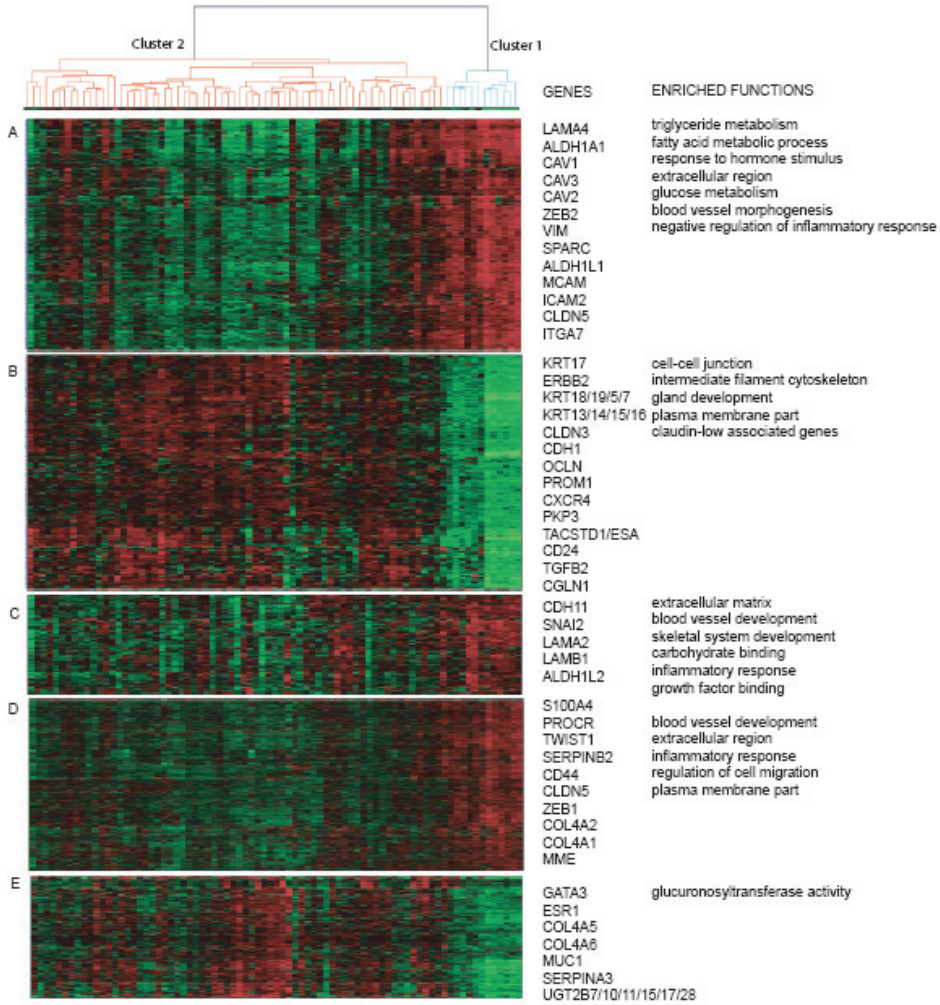


Figure 2: Selected genes from gene clusters up- and down-regulated in cluster 1. Gene functions/ontology terms associated with the respective gene clusters are given. In cluster 1 there is an up-regulation of mesenchymal genes and stem-cell related genes (A, C and D) and down-regulation of epithelial markers and claudins (B and E).

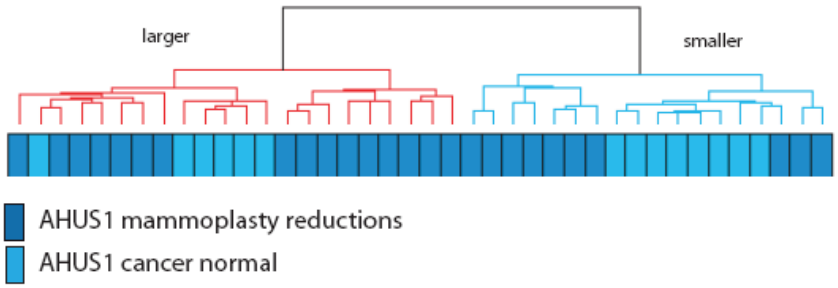


Figure 3: Unsupervised hierarchical clustering of 40 samples from the validation dataset AHUS1. Reduction mammoplasties and cancer normal samples are split between one larger and one smaller cluster, the smaller cluster containing slightly more mammoplasty reductions.

Supplementary material

Table S1 Gene ontology termes enriched in the genes differentially expressed between the two main samples in the MDG dataset (cluster 1 and 2 in Figure 1) and the AHUS1 dataset (small and large cluster in Figure 2)

	Cluster 1	AHUS1 small cluster
up	extracellular regioin	mitochondrion
	vascular development	response to hormone/insulin
	signal peptide	glucose metabolism
	regulation of lipid metabolism	vasculature development
	response to hormone/insulin	vitamin B6-binding
	glucose metabolic process	regulation of lipid metabolism
	triglyceride metabolism	carboxylic acid biosynthesis
	membrane organization	triglyceride metabolism
	protein dimerization	aerobic respiration
	regulation of inflammatory response	regulation of lipid storage
	cell motion	membrane
	organic acid biosynthesis	protein dimerization
	regulation of catabolic process	iron ion binding
	oxygen and reactive oxygen species	regulation of inflammatory response
	mitochondrion	
down	actin binding	adherens junction
	adherens junction	actin binding
	cell-cell junction	extracellular matrix
	cytoskeleton	cytoskeleton
	plasma membrane	cell-cell junction

Table S2 Gene set enrichment analysis (GSEA) of cluster 1 versus cluster 2 using selected gene lists from the literature. Significant FDR-values are marked red.

Gene list	FDR	Cluster 1
STROMA (FINAK)	**0.00**	Up
STROMA (CASEY)	**0.02**	Up
CD44+ (SHIPITSIN)	**0.01**	Up
BIPOLAR (VS LUM) (RAOUF)	0.10	Up
BIPOTENT (VS MYO) (RAOUF)	0.08	Up
MYOEPITHELIAL (JONES)	0.13	Up
IGS/CD44+ (LIU)	0.20	Up
EPITHIAL (FINAK)	**0.00**	Down
CD24+ (VS CD44) (SHIPITSIN)	**0.03**	Down
IGS/CD10 (LIU)	0.07	Down
RISK (CHEN)	0.24	Down
LUMINAL (JONES)	0.48	Down
EPITHIAL (JECHLINGER)	0.70	Down
MESENCHYMAL (JECHLINGER)	0.66	Down

Table S3 Comparison of cluster 1 and cell types/subtypes from published gene lists. The number of genes up- and down-regulated is given for genes characterizing each cell type and each cluster. Chi-squared test is used to illustrate the extent to which genes describing different cell types are equally regulated in the two clusters. The right column shows samples correctly identified by hierarchical clustering of the normal breast samples based on the gene list from the corresponding publication (see Supplemental file 1, Figure S2)

						Samples correctly identified by clustering	
Publication	Cell type	Method	up in cluster 1	down in cluster 1	cluster 1 resembles	Cluster 1	Cluster 2
Shipitsin, 2007	Epithelial	CD24+	132	152	CD44+	12/12	66/67
	Stem cell-like	CD44+	394	89			
	Unchanged		0	106			
	Sum		526	347			
	$\chi 2$	p-value	**2.2E-16**				
Jechlinger, 2003	Mesenchymal	Before and after TGFbeta-induced EMT	61	17	Mesen-chymal	11/12	67/67
	Epithelial		38	32			
	Unchanged		0	36			
	Sum		99	85			
	$\chi 2$	p-value	**6.9E-14**				

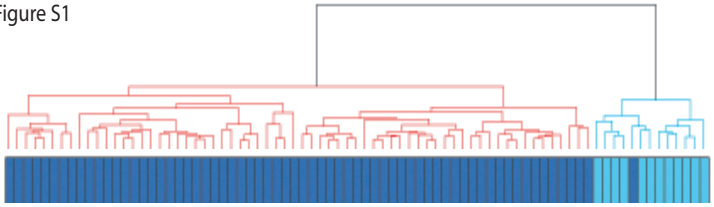
Table S3 cont

Publication	Cell type	Method	up in cluster 1	down in cluster 1	cluster 1 resembles	Samples correctly identified by clustering	
						Cluster 1	Cluster 2
Raouf, 2008	Luminal	CD49flow/ MUC1high/ CD10low	521	442	Progenitor	12/12	67/67
	Bipotent progenitor	CD49fhigh/ MUC1low/ CD10high	259	162			
	Unchanged		343	338			
	Sum		1123	942			
	$\chi 2$	p-value	**0.001**				
Raouf, 2008	Myoepithelial	CD49flow/ MUC1low/ CD10high	139	88	Progenitor	12/12	67/67
	Bipotent progenitor	CD49fhigh/ MUC1low/ CD10high	232	128			
	Unchanged		156	171			
	Sum		527	387			
	$\chi 2$	p-value	**2.40E-05**				
Liu, 2007	Stem cell-like	CD44+	50	17	Stem-like	11/12	67/67
	Epithelial	CD10+	34	58	Invasive		
	Unchanged		36	27	cells		
	Sum		120	102			
	$\chi 2$	p-value	**1.30E-05**				
Jones, 2004	Myoepithelial	MUC1+	65	91	-	12/12	66/67
	Luminal	CD10+	21	47			
	Unchanged		30	28			
	Sum		116	166			
	$\chi 2$	p-value	0.06				
Chen, 2009	IDC-like normal	Based on gene	11	81	Low risk	11/12	62/67
	Other normal c	expression	17	4			
	Unchanged		0	0			
	Sum		28	85			
	$\chi 2$	p-value	**1.3E-09**				
Finak, 2006	Stroma	Micro-	457	91	Stroma	12/12	67/67
	Epithelial	dissection	51	317			
	Unchanged		59	92			
	Sum		567	500			
	$\chi 2$	p-value	**2.2E-16**				

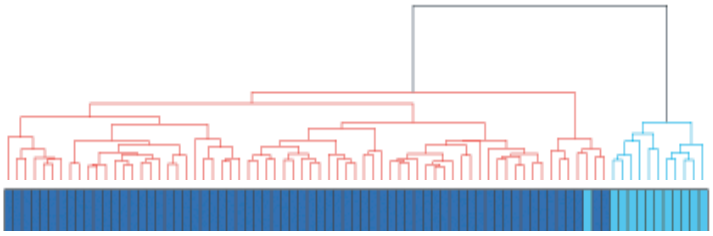
Table S3 cont

Publication	Cell type	Method	up in cluster 1	down in cluster 1	cluster 1 resembles	Samples correctly identified by clustering	
						Cluster 1	Cluster 2
Casey, 2008	Fibroblasts	Micro-dissection	330	140	Fibroblasts	11/12	64/67
	Epithelial		119	134			
	Unchanged		224	228			
	Sum		673	502			
	$\chi 2$	p-value	**1.9E-12**				
Villadsen, 2007	Stem-like	K19+/ K14+	71	104	Lineage restricted progenitor K19+/K14-	7/12	29/67
	Lineage restricted progenitors	K19+/ K14-	46	30			
		K19-/K14+	4	5			
		K19-/K14-	48	81			
	Unchanged		0	0			
	Sum		169	220			
	$\chi 2$	p-value	**0.008**				
Asztalos, 2010	nullipara	Micro-dissection	8	2	Post-pregnant		
	postpregnant		5	4			
	Unchanged		0	0			
Sorlie, 2001	Fisher		0.34		-	12/12	67/67
	Basal-like	Based on gene expression	62	66			
	HER2-enriched		72	69			
	Luminal A		73	80			
	Luminal B		70	60			
	Normal-like		80	91			
	Unchanged		0	0			
	Sum		357	366			
	$\chi 2$	p-value	0.76				
Hersch-kowitz, 2007	Basal-like	Based on gene expression	56	79	claudin-low	12/12	67/67
	Claudin-low		121	33			
	HER2-enriched		76	98			
	Luminal		70	95			
	Normal-like		76	67			
	Unchanged		163	116			
	Sum		562	488			
	$\chi 2$	p-value	**1.52E-12**				

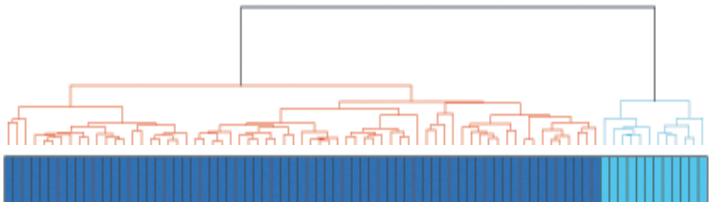
Figure S1



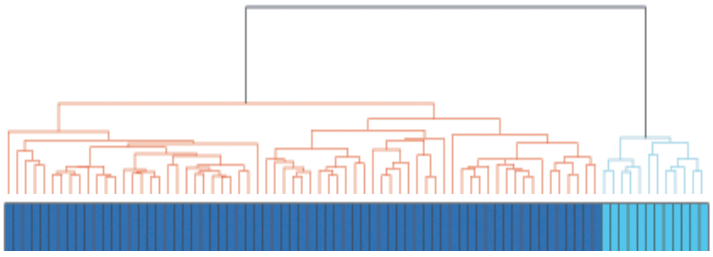
Shiptisin et al, 2007
Luminal (CD24+) vs
stem-cell like (CD44+) cells



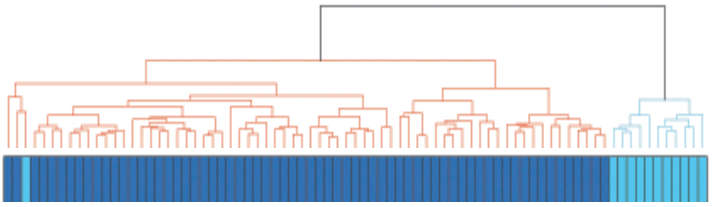
Jechlinger et al, 2003
Epithelial cells before and after
TGFbeta-induced EMT



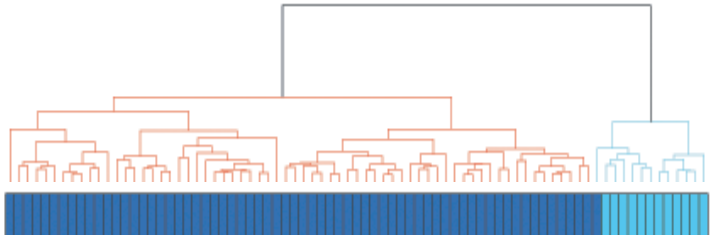
Raouf et al, 2008
Luminal (MUC1+ vs
bipotent (CD49f+, CD10+) cells



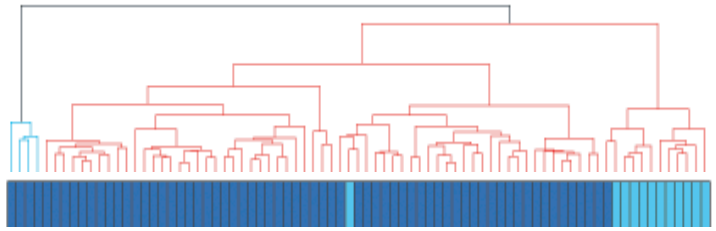
Raouf et al, 2008
Myoepithelial (CD10+) vs
bipotent (CD49+, CD10) cells



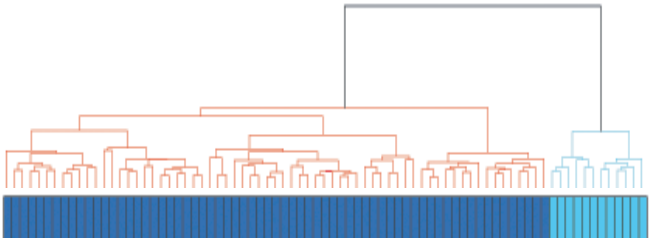
Liu et al, 2007
Stem-cell like (CD44+) vs
luminal (CD10+) cells



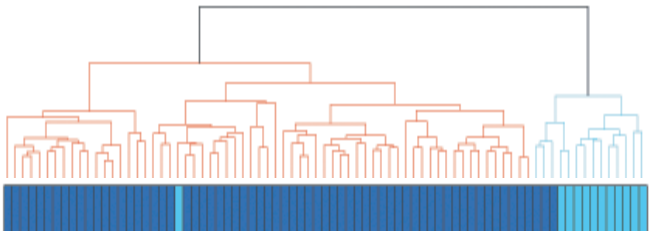
Jones et al, 2004
myoepithelial (MUC1+) vs
luminal (CD10+) cells

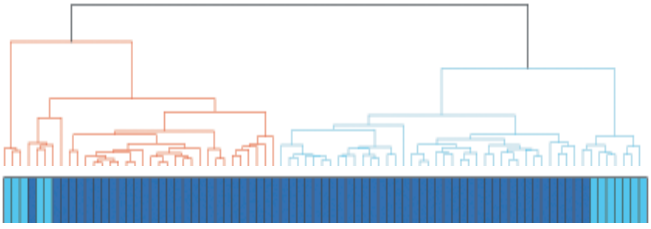


Chen et al, 2008
IDC-like normal cells vs
other normal cells

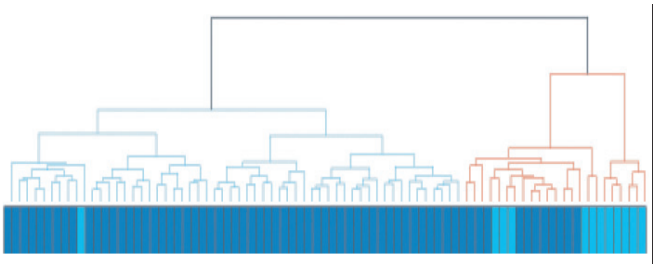


Finak et al, 2006
Stroma vs epithelial cells

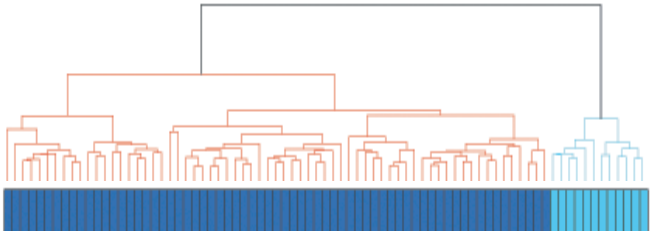




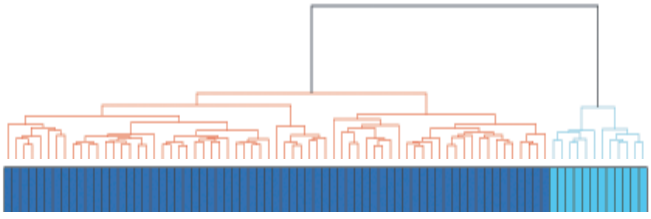
Villadsen et al, 2007
stem-cell like vs lineage
restricted progenitor cells



Asztalos et al, 2010
Nulliparous vs postpregnant women
Breast biopsies



Sorlie et al, 2001
Intrinsic genes



Prat et al, submitted
Claudin-low genelist

Hierarchical clustering of gene expression from 79 samples from breasts of healthy women. The samples are clustered based on gene lists from the literature, describing different cell types. The two last panels are clusterd based on gene lists used to identify breast cancer subtypes. Cluster 1-samples are marked light blue and cluster 2-samples dark blue. The dendrogram colors represent the two main clusters in the clustering performed based on the gene list in question.

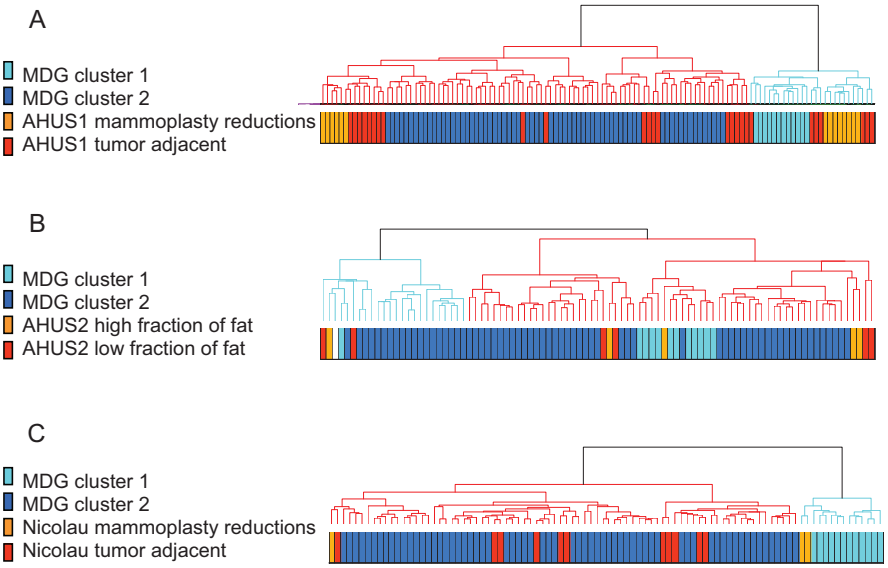


Figure S2 Biopsies from healthy women (MDG) clustered with two unpublished datasets from the hospital AHUS A) AHUS1 with breast biopsies from mammoplasty reductions (yellow) and tumor adjacent (red) tissue and B) AHUS2 with breast biopsies containing different known proportions of fat tissue and C) a dataset previously published by Nicoalu et al(1) with breast biopsies from mammoplasty reductions (yellow) and tumor adjacent (red) breast tissue. In all cases, the two datasets are merged by use of Distance Weighted Discrimination (DWD). This resulted in datasets with A) 8520 genes, B) 10078 and C) 3555 genes. Hierarchical clustering with Eucledian distance and Ward linkage was performed as described.

Reference list

1. Nicolau M, Tibshirani R, Borresen-Dale AL, Jeffrey SS: Disease-specific genomic analysis: identifying the signature of pathologic biology. Bioinformatics 2007; 23**:** 957-965.

Paper II

Expression levels of uridine 5′-diphosphoglucuronosyltransferase genes in breast tissue from healthy women are associated with mammographic density

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

Expression levels of uridine 5′-diphospho-glucuronosyltransferase genes in breast tissue from healthy women are associated with mammographic density

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Abstract

Introduction: Mammographic density (MD), as assessed from film screen mammograms, is determined by the relative content of adipose, connective and epithelial tissue in the female breast. In epidemiological studies, a high percentage of MD confers a four to six fold risk elevation of developing breast cancer, even after adjustment for other known breast cancer risk factors. However, the biologic correlates of density are little known.

Methods: Gene expression analysis using whole genome arrays was performed on breast biopsies from 143 women; 79 women with no malignancy (healthy women) and 64 newly diagnosed breast cancer patients, both included from mammographic centres. Percent MD was determined using a previously validated, computerized method on scanned mammograms. Significance analysis of microarrays (SAM) was performed to identify genes influencing MD and a linear regression model was used to assess the independent contribution from different variables to MD.

Results: SAM-analysis identified 24 genes differentially expressed between samples from breasts with high and low MD. These genes included three uridine 5′-diphospho-glucuronosyltransferase (*UGT*) genes and the oestrogen receptor gene (*ESR1*). These genes were down-regulated in samples with high MD compared to those with low MD. The UGT gene products, which are known to inactivate oestrogen metabolites, were also down-regulated in tumour samples compared to samples from healthy individuals. Several single nucleotide polymorphisms (SNPs) in the *UGT* genes associated with the expression of *UGT* and other genes in their vicinity were identified.

Conclusions: Three UGT enzymes were lower expressed both in breast tissue biopsies from healthy women with high MD and in biopsies from newly diagnosed breast cancers. The association was strongest amongst young women and women using hormonal therapy. UGT2B10 predicts MD independently of age, hormone therapy and parity. Our results indicate that down-regulation of *UGT* genes in women exposed to female sex hormones is associated with high MD and might increase the risk of breast cancer.

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Introduction

Breast cancer is a common disease in women. Knowledge about the first steps in tumour initiation is important for early detection. However, the exact mechanisms of tumour initiation are still unknown.

Mammographic density (MD), captured on film screen mammograms, refers to the content and architectural structure of the adipose, connective and epithelial tissues in the female breast [1]. In epidemiological studies, a high percentage of MD confers a four to six fold elevated risk of developing breast cancer [1-3] and has been proposed as a possible surrogate marker for the disease [4]. The relative risk associated with MDs remains at this magnitude even after adjustment for all other known breast cancer risk factors. Breasts with high MD have greater tissue cellularity and more tissue collagen [5]. Still, little is known as to how MD confers the increased breast cancer risk. MD is to a large degree an inherited trait, although it is also influenced by environmental factors, hormone therapy being an evident example [6]. The genetic factors determining the inheritability are largely unknown.

In order to elucidate how MD increases the risk of breast cancer; we searched for the biological correlates to MD. Gene expression analysis on biopsies from breasts of healthy women with varying degrees of MD was performed. The gene expression profiles represent the gene activity of the different cell types in the biopsy, producing a fingerprint of the breast tissue within the biopsy of that particular woman.

The breast is an oestrogen-sensitive organ. MD varies with levels of female hormones, and is reduced after menopause. The uridine 5'-diphospho-glucuronosyltransferase (*UGT*) genes encode enzymes inactivating several endogenous and exogenous compounds, including sex hormones (Figure 1) [7]. UGT1A1 is known to be responsible for the glucuronidation of bilirubin, but is also shown to glucuronidate catechol oestrogens [8,9]. Polymorphisms in this gene have previously been linked to MD in premenopausal women [10]. UGT2B7 is known to conjugate oestrone, one of the active oestradiol metabolites. This enzyme has previously been found to be down-regulated in tumour tissue compared with non-malignant tissue, leading to the conclusion that UGT expression could lead to the promotion of carcinogenesis [11] but there are no reports on this gene in relation to MD in the literature. Less is known about the other *UGT2B* genes, although there is extensive structural homology. We will use the *UGT* genes as a term describing three *UGT2B* genes significantly down-regulated in our analyses (*UGT2B7*, *UGT2B10* and *UGT2B11*). Other *UGT* genes are specified in the text. In this study we analysed biopsies from breasts of healthy women and found genes whose expression is associated with MD.

Materials and methods

Subjects

The women included in this study had all attended one of six breast diagnostic centres in Norway that are part of the governmentally funded National Breast Cancer Screening Program between 2002 and 2007 [12]. Women were eligible if they did not currently use

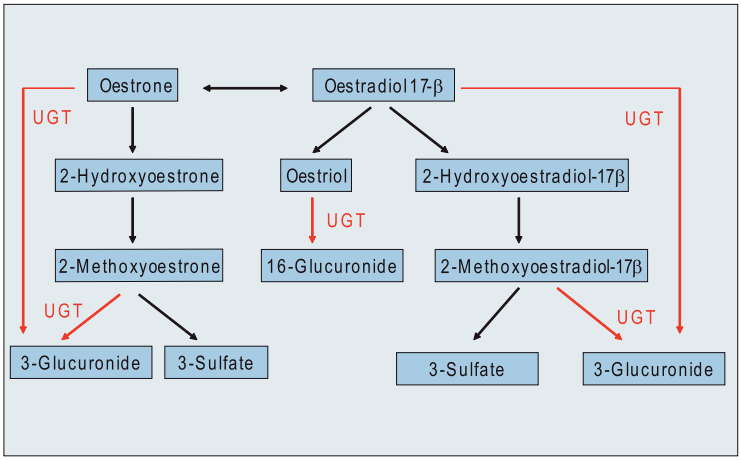

Figure 1 UGTs conjugate oestrogen-substrates into biologically inactive oestrogen glucuronides. The figure gives a schematic view with focus on glucuronidation and not a complete picture of oestradiol metabolism. Androgens are also inactivated by uridine 5'-diphospho-glucuronosyltransferases (UGTs), but are not included in this illustration.

anticoagulants, did not have breast implants and were not currently pregnant or lactating. A total of 186 women were recruited to the study; 120 healthy women with no malignant disease but some visible density in the mammograms, referred to here as healthy women, and 66 women with a newly diagnosed breast cancer. Of these, quality tested expression data were obtained from biopsies from 79 healthy women and 64 breast cancer patients.

The women were either referred to a breast diagnostic centre for a second look due to some irregularity of the initial screening mammogram (n = 69) or due to clinical findings (n = 83). For 34 women the type of referral was unknown.

The women provided information about height, weight, parity, hormone therapy use and family history of breast cancer. Two breast biopsies and three blood samples were collected from each woman. All women provided signed informed consent. The study was approved by the local ethical committee and local authorities (IRB approval no S-02036).

Core biopsies

Two breast biopsies were obtained from each woman with a 14 gauge needle, for RNA- and DNA-extraction. In healthy women, the biopsies were taken from an area with no visible pathology, but with some MD to ensure that the biopsies did not contain only fatty tissue, which yields little RNA. The sampling was guided by ultrasound. At one hospital, six of the biopsies from breasts of healthy women were collected from a benign lesion (mostly fibroadenomas). For the cancer patients, all biopsies were taken from the tumour. The tissue was either fresh-frozen at -80°C or soaked in ethanol and RNAlater (Ambion, Austin, TX, USA), transported and subsequently stored at -20°C.

Pathology

The haematoxylin eosinofil sections from the tumours of the breast cancer patients were evaluated locally and then re-evaluated by one pathologist (YC). Information about tumour size, histological grade and type, oestrogen and progesterone receptor status, human epidermal growth factor receptor (HER) 2 status and sentinel node status was recorded and entered into a database managed by the Office for Clinical Research at Oslo University Hospital, Radiumhospitalet. Pathology evaluations were not available for the biopsies from breasts of healthy women.

RNA-expression analysis

Homogenisation, cell lysis and RNA extraction were performed using the RNeasy Mini Protocol (Qiagen, Valencia, CA, USA). RNA quality was controlled by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and concentration was determined using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of 40 samples, mostly from normal breast tissue, were excluded from further analyses due to a low RNA amount (< 10 ng) or poor RNA quality. RNA was then amplified and labelled using the Agilent Low RNA input Fluorescent Linear Amplification Kit Protocol. Amplified tumour RNA was labelled by Cy5 (Amersham Biosciences, Little Chalfont, England, UK) and amplified RNA from Universal Human total RNA (Stratagene, La Jolla, CA, USA) was labelled by Cy3 (Amersham Biosciences, Little Chalfont, England, UK). RNA from the remaining 146 biopsies was further hybridised on Agilent Human Whole Genome Oligo Microarrays (G4110A) (Agilent Technologies, Santa Clara, CA, USA). Three arrays had to be excluded due to poor quality leaving data from 143 subjects (79 healthy individuals and 64 breast cancer patients) for further analysis. Of the 79 biopsies from healthy women, 5 had been obtained from a benign lesion. By ultrasound and mammography these 5 were described as fibroadenoma (n = 4) or microcalcification (n = 1).

RNA-data processing

The microarrays were scanned by an Agilent scanner (Agilent Technologies, Santa Clara, CA, USA) and processed in Feature Extraction 9.1.3.1 (Agilent Technologies, Santa Clara, CA, USA). Locally weighted scatterplot smoothing (lowess) was used to normalise the data. The normalised and log2-transformed data were stored in the Stanford Microarray Database [13] and retrieved from the database for further statistical analyses. Flagged spots were treated as missing values. The dataset now counted 40,791 probes. Clone IDs with 20% or more missing values were excluded. Gene filtering was performed to include only probes with variation across samples, so that probes with less than three arrays being at least 1.6 standard deviations from the mean were excluded. For the 79 healthy women, this probe filtration resulted in an expression dataset of 9,767 probes and 79 arrays each representing one individual. For the breast cancer women, a dataset of 64 arrays and 10,153 probes were obtained after filtration, and for both groups combined, a dataset of 143 arrays and 13,699 probes were obtained. Missing values were imputed in R using the method impute.knn in the library impute [14].

Genotyping

Blood DNA was extracted by phenol/chloroform extraction followed by ethanol precipitation (Nuclear Acid Extractor 340A; Applied Biosystems, Foster City, CA,

USA) according to standard procedures. *UGT* genotype data was retrieved from two sources: genome wide association studies (GWAS) using the Human-1 109K BeadChip (Illumina Inc, San Diego, CA, USA) and candidate gene-based study using iPlex, Sequenom. For the GWAS, each sample was subject to whole genome amplification using Illumina proprietary reagents [15]. The amplified DNA was fragmented and hybridised according to the protocol. The BeadArray reader (Illumina Inc, San Diego, CA, USA) with the BeadScan software (Illumina Inc, San Diego, CA, USA) was used to image the beadchips. Non-polymorphic probes and probes with more than 20% missing values and were excluded and data processed as described previously [16]. The candidate gene single nucleotide polymorphism (SNP) analyses were performed using the iPLEX assay in conjunction with the Sequenom MassARRAY platform. Multiplexing was performed in 384 plates using 1 ul DNA per well with one well containing up to 29 reactions. The technology is described in detail on the sequenom web-page [17].

Mammograms

Routine descriptions of mammograms by local radiologists were collected. Craniocaudal mammograms of both breasts were digitised using a high-resolution Kodak Lumisys 85 scanner (Kodak, Rochester, NY, USA). Density was quantified using the University of Southern California Madena assessment method [18]. In brief, the method works as follows: a reader (trained by GU) outlines the total area of the breast using a computerised tool, the software then counts the number of pixels. This represents the total breast area. MD is assessed (by GU), first by identifying a region of interest that incorporates all dense areas except those representing the pectoralis muscle and scanning artifacts, and then by applying a yellow tint to all pixels within the region of interest shaded at or above a threshold intensity of gray. The software then counts the tinted pixels, which represents the area of absolute density. The percent density is the absolute density area divided by the total breast area and is the value used for these analyses. Test-retest reliability was 0.99 for absolute density.

Statistical analysis

Clustering was performed using MatLab (version R2007b) (The MathWorks Inc., Natick, MA, USA) with Ward linkage and Euclidean distances. Before clustering, the data were gene centred, that is, for every probe the mean expression across all samples was calculated and was subtracted from the log2-ratios for that gene. This was performed for visualisation purposes only, clustering with uncentred data returns the same clusters. Significance analysis of microarrays (SAM, Stanford University, CA, USA) (version 3.02) [19,20] for Excel (Microsoft, Redmond, WA, USA) was used for analysis of differentially expressed genes between two groups of data. The data were not gene centred for the SAM analysis. A total of 500 permutations were used. Quantitative SAM analysis was used to identify genes differentially expressed according to MD as a continuous variable. Statistical significance tests and regression analysis were performed in R 2.9.0 [21]. To test for difference in the mean of phenotypic variables (MD, age, body mass index (BMI)) in different clusters of women, we used two-sided t-tests (assuming equal variance in the groups) and analysis of variance (ANOVA) for continuous variables and chi-squared/Fisher's exact tests for categorical variables [22]. To investigate the similarities of distributions of *UGT* genes between tumour samples and normal samples with low MD and high MD respectively, Kullback-Leibler distances between normalised distributions of the histograms of the data were calculated by use of MatLab (The MathWorks Inc., Natick, MA, USA). The cancer samples in our study were grouped into subtypes and assigned a risk group using the PAM50 gene list published by Parker et al [23]. SNP-analysis was performed using R 2.9.0 [21]. The association between gene expression and SNPs was assessed using expression quantitative trait loci (eQTL) [24]*in cis* (10^6 bp on each side of the gene) using the R package eMap v1.1 [25]. Comparing the akaike information criterion for different models predicting MD, the lower criterion singled out a linear regression model as the model fitting the distribution of the data best. A linear regression model was fitted in R 2.9.0 with MD as a continuous response variable and the following covariates: UGT2B7, two probes for UGT2B10, UGT2B11, ESR1, age, BMI, current hormone therapy, age at first birth and parity. Gene expression, age, age at first birth and BMI were entered into the model as continuous variables. Stepwise variable selection was performed, starting with all variables included in the model. For every step, the variable with the highest P value was rejected from the model and the model was refitted. This was repeated until all variables included in the model had a P value less than 0.05. To correct for the influence of age, this variable was forced to stay in the model. A sensitivity analysis was performed excluding extreme ages (30 years or younger) to check the robustness of the data. We also fitted linear regression stratified on age (younger or older than 50 years of age) and current use of hormone therapy. Gene ontology analysis was performed by the use of DAVID Bioinformatics Resources 2008 from the National Institute of Allergy and Infectious Diseases, NIH [26]. Functional annotation clustering was applied and the following gene ontology categories were selected: biological

processes (all), molecular function (all) and the KEGG pathway database. We included gene ontology terms with a *P* value (false discovery rate (FDR)-corrected) of less than 0.01 containing between 5 and 500 genes.

The normalised, log2-transformed data are available in Gene Expression Omnibus with accession number [GEO:GSE18672]. The data are not gene centered or gene filtered.

Results

Gene expression and mammographic density

To identify genes differentially expressed according to MD we performed quantitative SAM with MD as a continuous variable using gene expression data from the normal biopsies. Of 9,767 probes, only 25 probes, representing 24 genes, were differentially expressed according to MD, with reduced expression associated with higher MD (FDR < 25%; Table 1) [see Additional file 1]. Gene ontology analysis revealed no significant terms and we found no pathway associated with this gene set. The *UGT* genes and oestrogen receptor gene (*ESR1*) were among the genes significantly down-regulated in breasts with high MD. The percentage of samples with low UGT expression was higher in tumour samples than in normal samples with low MD, whereas the percentage was more similar between tumour samples and normal samples with high MD [see Figure S1 in Additional file 2]. The function of UGT-enzymes in oestradiol metabolism is illustrated in Figure 1. In healthy women, the expression of the different *UGT* genes was highly correlated with each other and the four probes clustered together [see Figures S2 and S3 and Table S1 in Additional file 2].

Table 1 Genes differentially expressed according to mammographic density in non-cancer samples

Gene symbol	Agilent ID	Cytogenetic band
729641	A_24_P932736	8p21.1
FLJ10404	A_23_P427472	5q35.3
VPS18	A_24_P18802	15q15.1
UGT2B10	A_23_P7342	4q13.2
CABP7	A_24_P177236	22q12.2
CD86	A_24_P131589	3q13.33
UGT2B11	A_23_P212968	4q13.2
580687	A_23_P152570	17p11.2
DIAPH2::RPA4	A_23_P254212	Xq21.33
LMOD1	A_32_P199824	1q32.1
UGT2B10	A_24_P521559	4q13.2
PIK3R5	A_23_P66543	17p13.1
ATG7	A_32_P107994	3p25.2
LRRC2	A_23_P155463	3p21.31
RBL1	A_23_P28733	20q11.23
NPY1R	A_23_P69699	4q32.2
810781	A_23_P144244	3q13.33
593535	A_32_P80016	15q26.1
H2AFJ	A_23_P204277	12p12.3
666399	A_32_P35668	20p12.3
Transcribed	A_24_P640617	2p25.2
Transcribed	A_32_P20997	20q13.13
UGT2B7	A_23_P136671	4q13
ESR1	A_23_P309739	6q25.1
SAPS1	A_23_P119448	19q13.42

MD was lower in women with BMI of 25 or more compared with those with BMI of less than 25 ($P = 0.01$), but unrelated to other epidemiological variables. UGT expression was not significantly associated with age, BMI, age at first birth or current hormone therapy use in the healthy women [see Table S2 in Additional file 2].

To dissect the impact of age and hormone therapy use, we performed SAM analyses to identify differentially expressed genes according to MD, whereas stratifying for age and postmenopausal hormone therapy use. For healthy women younger than 50 years of age, the *UGT* genes were not significant at a FDR of 25%. For healthy women aged 50 years or older, 49 probes were significantly down-regulated in breasts with MD of 30% or higher (FDR < 25%). Of these, 17 were overlapping with those significantly down-regulated among healthy women in the unstratified analysis. The *UGT* genes were not in this list. We then stratified the women aged 50 years or older on current hormone therapy use. When only those currently using hormone therapy were included in the analysis, *UGT2B7* and *UGT2B11* were among the six genes differentially expressed with an FDR less than 10E-5 and *UGT2B28* with FDR less than 25%. For healthy women above 50 years of age and not currently using hormone therapy, several of the 24 genes were differentially expressed according to MD with an FDR of less than 25%, but again the *UGT* genes were not in this list [see Additional file 3].

These analyses were confirmed fitting a linear regression model. Although the other variables were excluded from the model with insignificant *P* values, age was kept in the model to control for the age-effect. After stepwise variable selection, the only significant variables remaining in the model were UGT2B10 (A_23_P7342)($P = 0.005$) and BMI ($P = 0.015$). Sensitivity analysis excluding extreme ages (30 years and younger) did not alter the results (UGT2B10 $P = 0.003$, BMI $P = 0.016$) and indicates the robustness of the results. ESR1 was borderline significant in both these analyses. These results were not significantly altered when MD was log2-transformed. For further stratification see Table 2.

Unsupervised hierarchical clustering of the 79 samples from healthy women showed two main clusters. MD was not significantly different between these two clusters [see Figure S3 in Additional file 2].

Table 2 Linear regression analysis of factors predicting mammographic density in all women and stratified for age and hormone therapy use

Women in model	N	Variables	Beta value	*P* value
All women	76	UGT2B10[1)]	-0.6	0.902
		UGT2B7	1.8	0.631
		UGT2B11	4.8	0.275
		ESR1	-3.8	0.055
		UGT2B10[2)]	**-5.6**	**0.005**
		BMI	**-1.5**	**0.015**
		age	-0.4	0.074
50 years or older	46	UGT2B11	0.2	0.987
		UGT2B10[1)]	1.0	0.946
		UGT2B7	3.5	0.486
		UGT2B10[2)]	-3.7	0.073
		BMI	-1.4	0.052
		ESR1	**-6.0**	**0.016**
		age	-0.9	0.061
50 years or older, currently on hormone therapy	11	UGT2B10[1)]	7.2	0.771
		UGT2B11	-5.8	0.695
		BMI	-2.9	0.103
		UGT2B7	6.8	0.418
		UGT2B10[2)]	**-27.0**	**0.000**
		ESR1	**-8.1**	**0.011**
		age	-0.9	0.103
50 years or older, never used hormone therapy	28	UGT2B11	-0.7	0.948
		UGT2B10[1)]	3.3	0.809
		UGT2B7	3.1	0.555
		UGT2B10[2)]	-1.4	0.607
		BMI	-0.9	0.348
		ESR1	**-6.0**	**0.033**
		Age	**-1.5**	**0.004**
Younger than 50 years	30	UGT2B7	0.4	0.950
		UGT2B10[1)]	-1.2	0.866
		ESR1	-0.9	0.835
		UGT2B11	8.4	0.225
		BMI	-1.4	0.216
		UGT2B10[2)]	**-6.2**	**0.040**
		Age	-0.3	0.610

1) A_24_P521559, 2) A_23_P7342

Factors predicting mammographic density (MD) after stepwise exclusion of non-significant factors are shown. Variables listed in the order of exclusion from the model. *P* value from the last equation including the variable is shown. Age is forced to stay in the model. UGT2B10 (A_23_P7342) is a significant, independent predictor of MD in all analyses with a majority of women under influence of female hormones; women younger than 50 years of age and women currently on hormone therapy. BMI, body mass index.

In the breast cancer group, MD was significantly associated with age and BMI, with higher MD in the younger women and in those with BMI less than 25. Both MD and UGT expression tended to be higher in women with receptor positive tumours, but this was not significant for any type of receptor. UGT-expression in tumours was unrelated to age, BMI, age at first birth and current hormone therapy (data not shown). There was a higher proportion of oestrogen receptor positive tumours among the breast cancer patients with high MD (≥ 30%) compared with low (< 30%) MD (10 of 10 vs 36 of 40, Fisher's = 0.001). There was no significant association between tumour subtype and level of MD as assessed by ANOVA. There was no indication that degree of MD was associated with the risk of relapse as assessed by the method of Parker et al [23] [see Figure S4 of Additional file 2].

Nine probes were differentially expressed according to MD in cancer samples (FDR < 25%; Table 3). None of these were overlapping with the 24 genes differentially

Table 3 Genes differentially expressed according to mammographic density in cancer samples

Agilent ID	Gene name	FDR (%)
A_32_P171923	730402	0.00
A_32_P480177	TNN	0.00
A_23_P200298	AGL	0.00
A_24_P87036	TMEM16A	0.00
A_23_P312150	EDN2	14.87
A_23_P83388	EPPK1	14.87
A_32_P60065	F2RL2	19.82
A_32_P158272	MRNA	19.82
A_23_P105012	HRASLS2	19.82

FDR, false discovery rate.

expressed in the samples from the breasts of healthy women.

Genetic polymorphisms

In order to identify genetic determinants of the expression of the *UGT* genes found to be associated with MD, we performed eQTL analyses of SNPs in these genes as available from an array based GWAS study and a candidate gene study. Twenty one SNPs in *UGT* genes were present on the 109 K array from Illumina, and 9 SNPs from the candidate gene analysis. Of these, 5 SNPs were associated with the expression of *UGT* genes or other genes in their vicinity at $P = 0.05$ [see Additional file 4]. Two of these SNPs, both located in *UGT2B10* (rs1828705, rs1828705), were significantly associated with gene expression of another *UGT* gene (*UGT2B7* and *UGT2B28*).

Discussion

Previously, whole genome expression profiling of normal breast tissue (all cell types included) has been performed to a limited extent [27,28]. Yang et al recently performed a study of cancer-free breast tissue obtained from mastectomies in breast cancer patients with high and low MD [29]. They identified a list of 73 genes differentially expressed between high and low MD samples. Specifically, this included the down-regulation of several transforming growth factor (TGF) β-related genes in samples with high MD. In the present study we analysed breast biopsies from 79 healthy women and tumours of 64 women with breast cancer. Twenty-four genes were differentially expressed according to MD in the healthy samples. In breast tumours, none of these 24 genes were found differentially expressed according to MD. Tumour-specific deregulation of a large number of mRNA transcripts may be expected to overshadow the MD signature. In addition, the sample size is limited and the two sample sets (cases and controls) are not directly comparable with respect to MD [see Figure S5 in Additional file 2].

In our study, three *UGT* genes (*UGT2B11, UGT2B10* and *UGT2B7*) were differentially expressed according to MD in the breasts of healthy women. All these three enzymes had decreased expression in dense breasts. Previous knowledge links the UGT enzymes to the metabolism of female hormones known to influence the mammary glands (Figure 1). The over-representation of *UGT* genes on the list of significant genes along with a biological link makes these genes particularly interesting. In a linear regression model with age as a confounding factor, BMI and one of two probes for UGT2B10 were the only significant variables independently predicting MD, with ESR1 as a borderline significant covariate. The expression of these three *UGT2B* genes is highly correlated to each other and as expected only one probe remained in the regression model as an independent predictor of MD. BMI is known to be the strongest and most consistent epidemiological predictor of MD, and is expected to remain in the model. It is noteworthy that one of the *UGT* genes has an independent predictive value of a greater significance and magnitude than BMI. MD is determined by multiple factors. In a study of limited sample size, we can only expect to identify the strongest predictors.

UGT2B7 is postulated to protect the breast tissue from oestrogen metabolites locally [30], and this is consistent with our findings that breasts with higher MD have reduced expression of this gene. The main metabolites of oestradiol and oestrone (hydroxyl- and methoxy-oestrogen compounds) bind to the oestrogen receptor, but with a reduced affinity compared with oestradiol. UGT2B10 and 11 are not yet reported to be associated with MD or breast cancer, but UGT2B10 is involved in the metabolism of tobacco-related nitrosamines [31]. Less is known about UGT2B11. The different *UGT2B* genes are located close to each other on chromosome 4 and there is great homology between the genes [see Figure S6 in Additional file 2]. UGT1A1, previously linked to MD and breast cancer [32], is not represented on the microarray used in this study.

We have identified a set of genes differentially expressed according to MD. Interestingly, the *UGT* genes seem, to a greater extent than the other genes, to be more similarly expressed between tumour samples and normal samples from breasts with high MD as compared with normal samples from breasts with low MD [see Table S4 and Figure S7 in Additional file 2]. The other differentially expressed genes generally express the same levels in the tumours and in the biopsies from the healthy women with low MD. We cannot exclude that the *UGT* genes confer risk for breast cancer development through increasing MD, but further studies would be needed to investigate this.

We found the *UGT* genes to be differentially expressed in young women and women over 50 years of age currently on hormone therapy. SAM analysis of MD in women younger than 50 years did not give any differentially expressed genes with an FDR of less than 25%. However, several UGT-probes are on the top of the list of genes down-regulated in samples from breasts with high MD. The lack of significance could be due to low sample size (n = 30). As UGT enzymes conjugate oestradiol metabolites, its effect will be greater when there is an increased level of oestradiol present, whether the oestradiol is endogenous or exogenous. The linear regression analysis showed that UGT2B10 was predicting MD independent of age in all women, younger women and women older than 50 years currently using hormones. This leads to the hypothesis that decreased UGT expression in the breast of a woman with increased levels of female hormones confers an increased MD and possibly an increased risk of breast cancer.

The biology in breasts with high and low MD may differ, partly due to differences in proportion of fatty tissue. Therefore, we looked for differentially expressed genes in a subset of samples including only samples from breasts with MD of more than 20%. The fact that the *UGT2B* gene family is so strongly represented among the down-regulated genes (six probes representing five different *UGT2B* genes are the only genes differentially expressed with an FDR < 10E-5) indicate that reduced UGT expression is of greater significance in breasts with higher MD and lower content of fatty tissue.

We find that *ESR1* is down-regulated in biopsies from healthy women with high MD compared with those with low MD. This is not consistent with previous findings [33] and contrary to what one would expect because *ESR1* induces transcription and epithelial growth and high MD may contain increased amounts of epithelial cells [34,35]. However, increased levels of oestradiol have been shown to decrease levels of ESR1 in breast cancer [36], and in normal breast tissue in monkeys [37] and in mice [38]. Increased levels of oestradiol may increase MD. Elevated expression of ESR1 is common postmenopausally [37] and represents non-proliferating cells. The association between reduced levels of ESR1 and high MD may reflect high levels of oestradiol. We found that ESR1 was only a borderline significant predictor of MD in models with stepwise exclusion of covariates. In a model including ESR1 with only age or age and UGT2B10, ESR1 was significantly predicting MD. The independent contribution of ESR1 in predicting MD was significant in older women, where the effect of UGT2B10 was not present. There could be a link between UGT-expression and ESR1-expression in that reduced metabolism of oestradiol-metabolites increases the levels of ESR1-ligands (oestradiol metabolites) and hence reduces ESR1-levels. The UGT-enzyme activity may be the cause of the alterations leading to increased MD by this mechanism. Reduced ESR1 is only borderline significant in predicting MD and could also be an intermediate factor.

MD is the result of complex biological processes without any single determining factor. BMI is the single most important factor found to date, and is also significant in this study. Age seems to have its effect mainly through hormonal influence, except for in postmenopausal women not taking hormones, where age has a significant, independent effect on MD. MD is not significantly different between the two main clusters from unsupervised hierarchical clustering of the samples from healthy women. MD is hence not related to the main variation in the normal samples.

The genes whose expression we have found to be associated with MD do have a fairly high FDR in a SAM analysis and are not significant in all stratified analyses, suggesting that they may play a role in only subsets of individuals and other factors also have a significant contribution. Despite this, in linear regression models UGT2B10 is an independent predictor of MD along with BMI.

There is a substantial heritable proportion of MD. SNPs in *UGT* genes with influence on the UGT expression have been described [8,39]. We identified two UGT-SNPs associated with the expression of other *UGT* genes. Due to their homology and co-localisation on the chromosome, they may share common control loci that affect the expression of multiple *UGT* genes. It remains to be investigated in larger and better powered epidemiological studies whether any of these SNPs are associated to MD *per se*.

We do not know enough about the variability of gene expression within normal breasts to know if the genes relevant for MD are adequately represented by one biopsy taken from an area with some MD. It is previously shown that two biopsies from the same breast tumour, before and after chemotherapy, cluster together [40]. The tumours may, however, be more homogenous than normal breast tissue. Variability in gene expression within each breast will make it difficult to detect genes with only a minor influence on MD so that only the strongest factors are identified. In an unpublished dataset we found no significant difference between UGT-expression in tumours and normal adjacent tissue tested by paired t-test [see Table S5 in Additional file 2]. This is merely an indication that the expression in one breast might be similar for different locations in the breast and hence be used to look for associations with MD.

In this study, healthy individuals had higher MD than the breast cancer patients. The women recruited in the study had been referred to a breast diagnostic centre for a second look. As high MD confers an increased risk for breast cancer and mammograms with high MD are more difficult to interpret, they most likely had a higher MD. In addition, the inclusion criterion of some visible MD for biopsy may have influenced the mean MD of the study population. The two populations are not directly comparable with respect to MD and related parameters. This lack of comparability on MD does not affect the analyses of gene expression among the healthy women only.

We obtained good quality microarrays from only 79 of 120 healthy women and from 64 of 66 breast cancer patients. This was due to low mRNA-yield or low mRNA-quality. The biopsies from healthy women consistently yielded less mRNA than the tumour samples. There is significantly higher MD in the breasts of healthy women with successful microarrays than in those with unsuccessful microarrays (37% vs 29%, P = 0.03). As samples from breasts with low MD are underrepresented in the microarray study, it is more difficult to identify genes that are differentially expressed between breast tissue with high and low MD. Despite these limitations, we have identified differentially expressed genes. These genes might have a greater significance than shown in this study.

Normal breast tissue yields less RNA than tumour tissue. The biopsies in this study were small and in agreement with the pathologist, all tissue from normal breasts was prioritised for RNA-extraction rather than histological evaluation. Imprint was not in routine use in the hospitals where we started this study. In order to make it possible for the staff to include women in this study in a busy schedule we had to use procedures already established. We do therefore not have any information about the cell types of the normal biopsies. Knowledge about the cell types present in the biopsies would have facilitated the analysis.

The two UGT2B10-probes behave differently in our dataset. Both probes map to the 3'end of the UGT2B10-gene by BLAT (98.4% homology for A_23_P7342 and 100% homology for A_24_P521559). The discrepancy in UGT2B10-expression detected by the two probes may be due to the fact that they both also share substantial sequence homology with other, but different UGT2B-genes.

Conclusions

We have identified a set of genes that are differentially expressed according to MD in breast samples from healthy women. Some of these genes are known to influence MD and breast cancer, such as *ESR1* and *UGT2B7*. Two less described *UGT* genes, *UGT2B10* and *UGT2B11*, are also differentially expressed. The expression of the three *UGT* genes is reduced in samples with high MD and also in tumour samples, but does not vary between different tumour subtypes or risk groups. The UGT enzymes are known to conjugate active oestrogen-metabolites. We show that UGT2B10 expression and BMI are independent predictors of MD. The influence of reduced UGT expression was strongest in women under exposure of female hormones. Two candidate SNPs are associated with the *UGT* gene expression *in cis*. We hypothesise that reduced expression of *UGT* genes in women exposed to female sex hormones, increase MD and that this may be associated with an increased risk of breast cancer. Further studies of these genes are needed to test the hypothesis that the gene products from these genes protect the breast from the oestrogen-induced MD and thereby reducing the risk of breast cancer.

Additional material

Additional file 1: Healthy SAM MD. Significance analysis of microarrays (SAM) for genes differentially expressed according to mammographic density (MD).

Additional file 2: Figures and tables. A collection of figures and tables describing the data set and the uridine 5'-diphospho-glucuronosyltransferase (UGT) genes. The main text refers to individual figures and tables in this file.

Additional file 3: Healthy SAM MD stratified. Significance analysis of microarrays (SAM) for genes differentially expressed according to mammographic density (MD) stratified on age and use of hormone therapy.

Additional file 4: eQTL. Expression quantitative trait loci (eQTL) analysis of single nucleotide polymorphism (SNPs) affecting the expression of uridine 5'-diphospho-glucuronosyltransferase (UGT) genes *in cis*.

Abbreviations

ANOVA: analysis of variance; BMI: body mass index; eQTL: expression quantitative trait loci; ESR1: oestrogen receptor; FDR: false discovery rate; GWAS: genome wide association studies; HER: human epidermal growth receptor; MD: mammographic density; SAM: significance analysis of microarrays; SNP: single nucleotide polymorphism; UGT: uridine 5'-diphospho-glucuronosyltransferase.

Acknowledgements

This study was funded primarily by The Research Council of Norway and South-Eastern Norway Regional Health Authority. We thank all the women who participated in the study and all the personnel in the hospitals who made the inclusion of these women possible, in particular the responsible radiologists: Einar Vigeland, Rolf O Næss and Else Berit Velken. We would also like to thank Lars Ottestad for help in the initiation of the project, Hilde Johnsen, Caroline Jevanord Frøyland and Marit Hilsen for lab assistance and to Eunjung Lee for statistical help. No medical writers were involved in this paper.

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Authors' contributions

The trial was designed by ALBD, ÅH, GU, MMH and VNK. ALBD and ÅH ensured funding. MMH, JOF, DN, LR, IKB and VDH assisted in data collection. MB and VNK are responsible for SNP analyses. VDH and TL contributed to the laboratory work. GU estimated the amount of mammographic density. OCL, VDH, HKS and MB performed statistical analyses of the data. ÅH, ALBD and VDH interpreted the results and wrote the paper. All authors were involved in reviewing the report.

Competing interests

The authors declare that they have no competing interests.

Received: 5 August 2010 Revised: 5 August 2010
Accepted: 27 August 2010 Published: 27 August 2010

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Cite this article as: Haakensen *et al.*: **Expression levels of uridine 5'-diphospho-glucuronosyltransferase genes in breast tissue from healthy women are associated with mammographic density.** *Breast Cancer Research* 2010 **12**:R65.

Additional file 1 - SAM MD, healthy women

Current settings

Input parameters

Data type?	Quantitative
Arrays centered?	USANN
Delta	0.1128214
Minimum fold change	0
Test statistic	standard
Regression method	standard
Are data are log scale?	USANN
Number of permutations	500
Input percentile for exchangeability factor s0	Automatic
Number of neighbors for KNN	10
Seed for Random number generator	1234567

Computed values

Estimate of pi0 (proportion of null genes)	0.9931402
Exchangibility factor s0	0.0010565
s0 percentile	0
False Discovery Rate (%)	50.528184

List of Significant Genes for Delta = 0.113

Positive genes (3)

Row	Gene ID	Gene Nam	Score(d)	Numerator	Denominator(s+	q-value(%)
4650	A_24_P756	LOC730057	2.8605802	0.015238	0.005326743	34.05052
8807	A_24_P329	BTN3A1	2.8288494	0.011028	0.003898406	34.05052
9112	A_24_P311	BTN3A3	2.7601417	0.009886	0.00358167	34.05052

Negative genes (54)

Row	Gene ID	Gene Nam	Score(d)	Numerator	Denominator(s+	q-value(%)	FDR<25
6760	A_24_P932	729641	-2.939794	-0.024561	0.008354769	0	
6129	A_23_P427	FLJ10404	-2.898094	-0.017947	0.006192794	0	
9647	A_24_P188	VPS18	-2.874372	-0.020757	0.007221398	0	
4530	A_23_P734	UGT2B11	-2.870324	-0.020651	0.007194816	0	
6993	A_24_P177	CABP7	-2.868285	-0.025244	0.008800965	0	
9508	A_24_P131	CD86	-2.834444	-0.025353	0.00894473	0	
2950	A_23_P212	UGT2B11	-2.791517	-0.029178	0.010452204	0	
3485	A_23_P152	580687	-2.7432	-0.020484	0.007467054	0	
7883	A_23_P254	DIAPH2::RI	-2.68794	-0.020478	0.0076185	8.276168	
8366	A_32_P199	LMOD1	-2.675115	-0.023358	0.008731604	8.276168	
7637	A_24_P521	UGT2B10	-2.656355	-0.023386	0.008803889	8.276168	
2978	A_23_P665	PIK3R5	-2.655412	-0.017592	0.006625019	8.276168	
6371	A_32_P107	ATG7	-2.590986	-0.021892	0.008449193	8.276168	
5506	A_23_P155	LRRC2	-2.573214	-0.019142	0.007439063	15.681161	
4399	A_23_P287	RBL1	-2.537519	-0.009507	0.003746516	15.681161	
1537	A_23_P696	NPY1R	-2.536482	-0.025025	0.009866086	15.681161	
8728	A_23_P144	810781	-2.511297	-0.018144	0.007224783	15.681161	
4605	A_32_P800	593535	-2.510476	-0.019579	0.007798728	15.681161	
4562	A_23_P204	H2AFJ	-2.505133	-0.011728	0.00468148	15.681161	
5316	A_32_P356	666399	-2.481846	-0.016741	0.006745418	15.681161	
6564	A_24_P640	Transcribed	-2.434159	-0.016788	0.006896857	18.916956	
6272	A_32_P209	Transcribed	-2.418337	-0.011417	0.004721011	21.590004	
2288	A_23_P136	UGT2B7	-2.403406	-0.029123	0.012117417	21.590004	
3534	A_23_P309	ESR1	-2.388786	-0.017364	0.007268849	21.590004	
2471	A_23_P119	SAPS1	-2.365048	-0.016886	0.007139654	23.835364	
9547	A_24_P125	PPM1F	-2.329502	-0.013608	0.00584143	26.738389	

8792	A_24_P180	UGT2B28	-2.294363	-0.023268	0.010141551	28.375433
7921	A_24_P693	ZNF552	-2.29271	-0.014196	0.006191895	28.375433
9066	A_24_P368	RAP1GAP	-2.277258	-0.014689	0.006450451	30.821591
9523	A_24_P287	PLCB2	-2.264593	-0.013956	0.006162608	33.104672
7706	A_24_P178	LOC13220	-2.251632	-0.010829	0.004809546	33.104672
3043	A_23_P256	EEF1A2	-2.245056	-0.009618	0.004283879	33.104672
4986	A_24_P176	UGT2B17	-2.191409	-0.017356	0.007920074	40.262439
2186	A_23_P902	CHST8	-2.184548	-0.011603	0.00531145	40.262439
3978	A_23_P436	OSTbeta	-2.173318	-0.015595	0.007175527	40.262439
6354	A_24_P844	710943	-2.154978	-0.010026	0.004652441	40.744212
8645	A_24_P734	CDNA	-2.139041	-0.011402	0.005330588	40.744212
8396	A_24_P913	797019	-2.126743	-0.013371	0.006287243	40.744212
4352	A_32_P163	NFE2L1	-2.122614	-0.00828	0.003900752	40.744212
27	A_32_P149	537146	-2.121891	-0.011736	0.005530906	40.744212
8825	A_24_P872	HIST2H2A	-2.116853	-0.007738	0.003655275	40.744212
9195	A_24_P315	825337	-2.103838	-0.011041	0.005248156	44.139563
2652	A_32_P595	GFRA1	-2.089581	-0.010792	0.005164611	44.139563
9608	A_24_P585	837185	-2.089248	-0.008928	0.004273281	44.139563
9565	A_24_P575	835938	-2.085134	-0.013291	0.006374112	44.139563
4339	A_23_P556	SLC14A1	-2.080633	-0.010976	0.005275552	44.139563
3525	A_23_P140	KRT8	-2.073511	-0.0086	0.00414763	44.139563
5841	A_24_P171	DKFZP547	-2.072369	-0.014788	0.007135751	44.139563
6833	A_23_P102	A_23_P102	-2.066516	-0.012184	0.005895961	44.139563
1659	A_23_P428	HIST1H2A	-2.062726	-0.009283	0.004500294	44.139563
2325	A_23_P407	OSTalpha	-2.060191	-0.009576	0.004648155	44.139563
6618	A_23_P735	CITED1	-2.048192	-0.014632	0.007143821	47.851299
7156	A_24_P464	RBM22	-2.039381	-0.015274	0.007489543	48.770276
7002	A_23_P384	SLC45A4	-2.021144	-0.012841	0.006353311	50.528184

Estimated Miss rates for Delta=0.112821421147983

Quantiles	Cutpoints	Miss Rate(%)
0 -> 0.05	-2.012 -> -1.228	0
0.05 -> 0.1	-1.228 -> -0.969	3,49
0.1 -> 0.15	-0.969 -> -0.783	0
0.15 -> 0.2	-0.783 -> -0.633	0
0.2 -> 0.25	-0.633 -> -0.5	0
0.25 -> 0.75	-0.5 -> 0.533	0,44
0.75 -> 0.8	0.533 -> 0.654	8,29
0.8 -> 0.85	0.654 -> 0.807	0,71
0.85 -> 0.9	0.807 -> 1.002	0,76
0.9 -> 0.95	1.002 -> 1.254	11,81
0.95 -> 1	1.254 -> 2.505	0

Additional file 2 - Figures and tables

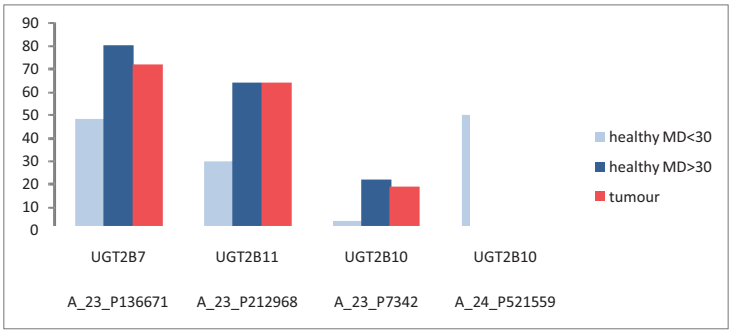


Figure S1: The percentage of samples with low expression of *UGT* genes (<-0.5) within tumour samples and healthy women with high (≥30%) or low (<30%) MD.

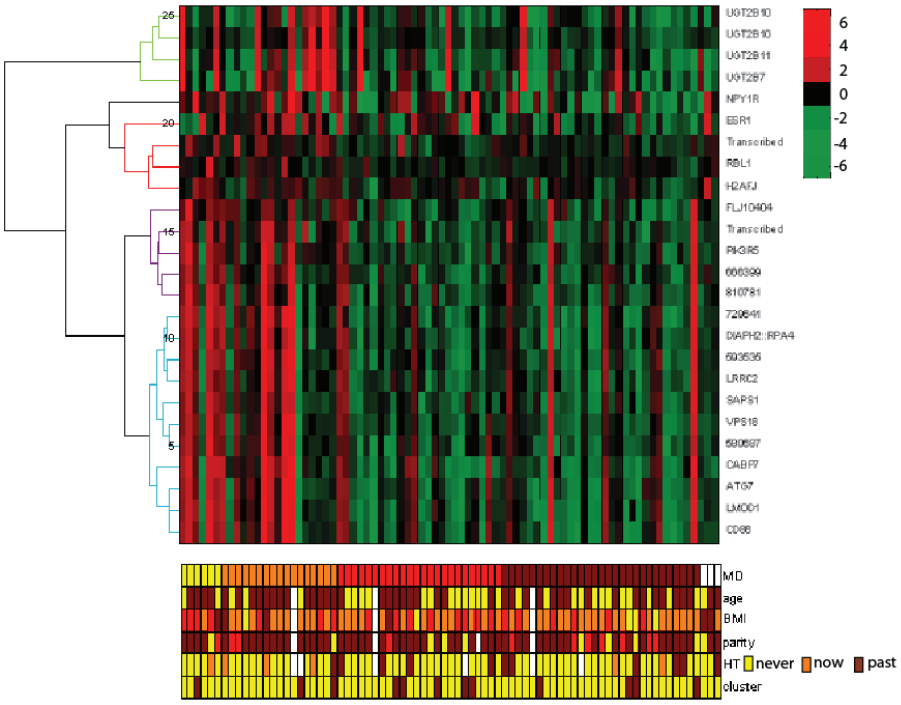


Figure S2: Clustering of the genes significantly down regulated in high MD samples. The three UGT genes cluster separately and tightly together. Samples are sorted according to MD.

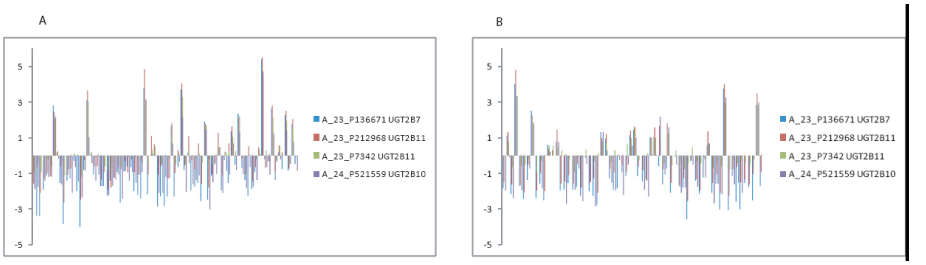


Figure S3: The expression of the different *UGT* genes is highly correlated. Expression of the four probes representing UGT-transcripts (y-axis) for each sample (x-axis) for A) healthy women and B) breast cancer patients respectively

Table S1: Correlation between the expression of different UGT genes

		A_23_P136671 UGT2B7	A_23_P212968 UGT2B11	A_23_P7342 UGT2B10	A_24_P521559 UGT2B10
A_23_P136671	UGT2B7	-	0.92	0.91	0.90
A_23_P212968	UGT2B11	0.92	-	0.93	0.94
A_23_P7342	UGT2B10	0.91	0.93	-	0.90
A_24_P521559	UGT2B10	0.90	0.94	0.90	-

Table S2: MD and expression of UGT genes in samples from healthy women

		MD	A_23_P136671 UGT2B7 expression	A_23_P7342 UGT2B10 expression	A_24_P521559 UGT2B10 expression	A_23_P212968 UGT2B11 expression
age	mean <50 (n=31)	40.4	-0.83	0.15	-0.74	-0.07
	mean ≥50 (n=45)	35.2	-0.68	0.22	-0.70	-0.07
	p-value	0.25	0.72	0.78	0.92	0.99
BMI	mean <25 (n=49)	41.2	-0.84	0.12	-0.77	-0.11
	mean ≥25 (n=27)	30.0	-0.57	0.31	-0.63	0.02
	p-value	0.01	0.53	0.45	0.66	0.73
Age at first birth	mean <25 (n=38)	31.5	-0.66	0.25	-0.60	-0.03
	mean ≥25 (n=18)	39.0	-0.64	0.22	-0.48	0.00
	p-value	0.14	0.96	0.93	0.77	0.95
Current hormone therapy	mean yes (n=64)	46.5	-0.97	-0.12	-1.04	-0.50
	mean no (n=12)	35.6	-0.71	0.24	-0.64	0.01
	p-value	0.07	0.65	0.25	0.35	0.30

MD and expression of UGT genes in samples from healthy women in relation to epidemiological factors. All p-values are from two-sided t-tests not corrected for multiple testing.

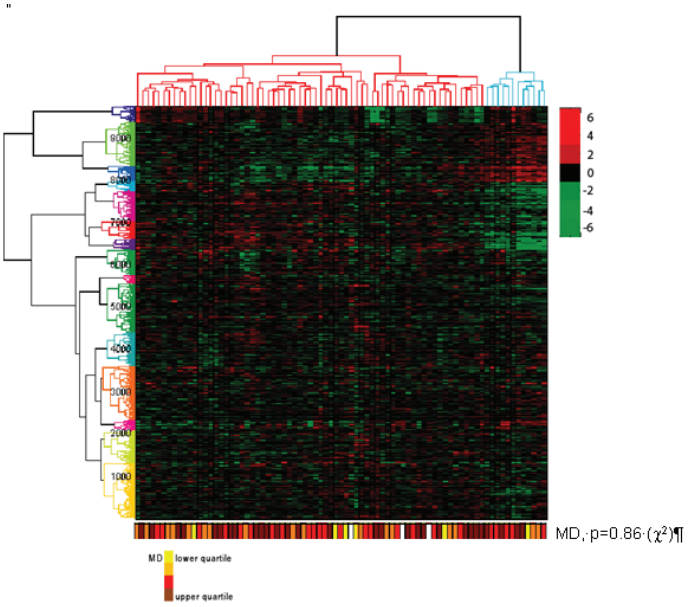
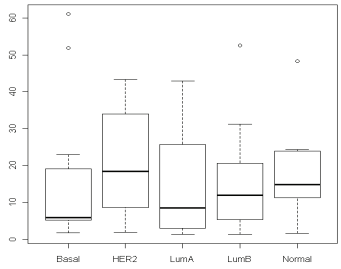


Figure S3: Unsupervised hierarchical clustering showed two main clusters. MD was not significantly different between these two clusters.

Figure S4: MD in relation to tumour subgroups

a) Boxplot of MD vs subtypes



b) Boxplot of MD for each pam50 risk group (1=high, 3=medium, 5=low risk).

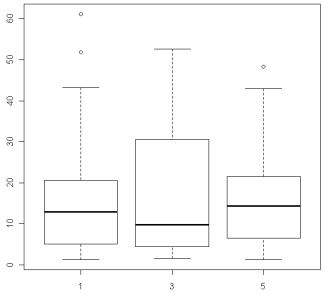
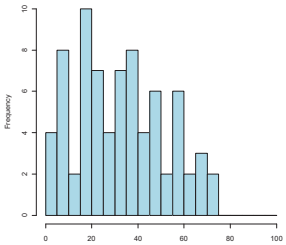
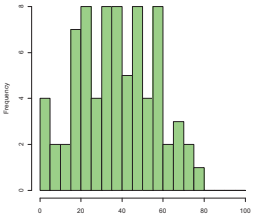


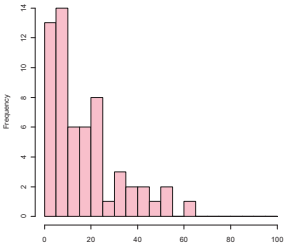
Figure S5: Distribution of MD in a) all samples (mean=), b) healthy women (mean=37%) and c) breast cancer patients (mean=16%)



a) MD frequency all women



b) MD frequency healthy women



c) MD frequency BC patients

Figure S6: Localisation of UGT genes on chromosome 4

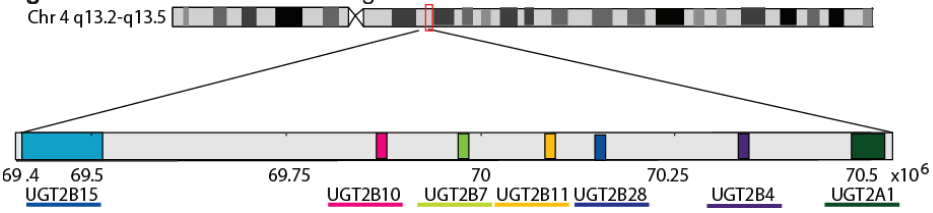


Table S4: Gene expression in samples from healthy women with differing MD compared with tumour samples.

A) MD < 30% vs MD>30%: The mean expression of all four probes representing *UGT* genes is not significantly different between tumour samples and normal samples from breasts with high MD. For three UGT probes, mean expression is significantly different between tumour samples and normal samples from breasts with low MD. This is not the case for most other genes.

Agilent ID	SYMBOL	mean t	mean n MD <30%	mean n MD>30%	ttest t vs MD<30%	ttest t vs n MD>30%
A_23_P119448	SAPS1	0.87	0.98	0.13	0.693	0.000
A_23_P136671	**UGT2B7**	**-1.00**	**0.21**	**-1.24**	**0.005**	**0.402**
A_23_P144244	810781	1.16	1.12	0.28	0.889	0.000
A_23_P152570	580687	1.53	1.78	0.87	0.430	0.003
A_23_P155463	LRRC2	1.39	1.60	0.65	0.478	0.001
A_23_P204277	H2AFJ	1.62	1.20	0.79	0.047	0.000
A_23_P212968	**UGT2B11**	**-0.43**	**0.77**	**-0.50**	**0.003**	**0.768**
A_23_P254212	DIAPH2::RPA4	1.50	1.62	0.68	0.705	0.000
A_23_P28733	RBL1	-0.90	-0.77	-1.12	0.306	0.010
A_23_P309739	ESR1	2.32	1.96	1.32	0.282	0.000
A_23_P427472	FLJ10404	1.21	1.44	0.51	0.466	0.002
A_23_P66543	PIK3R5	0.89	0.95	0.07	0.833	0.000
A_23_P69699	NPY1R	1.42	2.01	1.35	0.267	0.902
A_23_P7342	**UGT2B10**	**0.16**	**0.79**	**-0.13**	**0.010**	**0.054**
A_24_P131589	CD86	1.57	1.93	0.79	0.305	0.002
A_24_P177236	CABP7	1.63	1.95	0.68	0.378	0.000
A_24_P18802	VPS18	1.44	1.64	0.69	0.510	0.000
A_24_P521559	**UGT2B10**	**-0.70**	**0.07**	**-1.08**	**0.072**	**0.148**
A_24_P640617	Transcribed	0.97	1.04	0.18	0.801	0.000
A_24_P932736	729641	1.55	1.68	0.59	0.714	0.000
A_32_P107994	ATG7	1.51	1.78	0.74	0.413	0.001
A_32_P199824	LMOD1	1.55	1.91	0.77	0.320	0.002
A_32_P20997	Transcribed	0.25	0.56	0.10	0.008	0.092
A_32_P35668	666399	1.31	1.51	0.62	0.499	0.001
A_32_P80016	593535	1.59	1.72	0.75	0.676	0.000

Table S4 cont B) MD<20% vs MD>40%: The mean expression of all four probes representing UGT genes is not significantly different between tumour samples and normal samples from breasts with high MD, as opposed to most other probes. There is no significant difference in mean expression between tumour samples and normal samples from breasts with low MD. Contrary to most other probes, the mean expression of the UGT genes in tumours is more similar to the mean expression in normal samples from breasts with high than low MD.

			MD <20% vs >40%							
Agilent ID	SYMBOL	mean t	mean n MD <20%	mean n MD >40%	ttest t vs n MD <20%	ttest t vs n MD >40%	t-n low MD	|t-n low MD|	t-n high MD	t closer to
A_23_P119448	SAPS1	0.87	1.31	0.18	0.23	0.01	-0.44	0.44	0.69	low
A_23_P136671	**UGT2B7**	**-1.00**	**-0.39**	**-1.22**	**0.22**	**0.52**	**-0.61**	**0.61**	**0.21**	**high**
A_23_P144244	810781	1.16	1.46	0.37	0.42	0.00	-0.31	0.31	0.79	low
A_23_P152570	580687	1.53	2.12	0.98	0.13	0.04	-0.59	0.59	0.55	high
A_23_P155463	LRRC2	1.39	1.78	0.72	0.29	0.01	-0.39	0.39	0.67	low
A_23_P204277	H2AFJ	1.62	1.18	0.73	0.11	0.00	0.44	0.44	0.89	low
A_23_P212968	**UGT2B11**	**-0.43**	**0.24**	**-0.52**	**0.15**	**0.75**	**-0.66**	**0.66**	**0.09**	**high**
A_23_P254212	DIAPH2/RPA4	1.50	1.98	0.75	0.23	0.01	-0.48	0.48	0.76	low
A_23_P28733	RBL1	-0.90	-0.70	-1.15	0.18	0.01	-0.20	0.20	0.26	low
A_23_P309739	ESR1	2.32	1.89	1.21	0.32	0.00	0.43	0.43	1.12	low
A_23_P427472	FLJ10404	1.21	1.70	0.53	0.24	0.01	-0.49	0.49	0.68	low
A_23_P66543	PIK3R5	0.89	1.14	0.17	0.51	0.01	-0.25	0.25	0.72	low
A_23_P69699	NPY1R	1.42	2.21	1.26	0.25	0.73	-0.78	0.78	0.16	high
A_23_P7342	**UGT2B10**	**0.16**	**0.50**	**-0.15**	**0.20**	**0.07**	**-0.34**	**0.34**	**0.30**	**high**
A_24_P131589	CD86	1.57	2.26	0.87	0.10	0.02	-0.68	0.68	0.70	low
A_24_P177236	CABP7	1.63	2.25	0.77	0.17	0.01	-0.62	0.62	0.86	low
A_24_P18802	VPS18	1.44	1.88	0.77	0.23	0.01	-0.44	0.44	0.67	low
A_24_P521559	**UGT2B10**	**-0.70**	**-0.38**	**-1.14**	**0.52**	**0.17**	**-0.32**	**0.32**	**0.44**	**low**
A_24_P640617	Transcribed	0.97	1.25	0.32	0.42	0.01	-0.28	0.28	0.65	low
A_24_P932736	729641	1.55	2.10	0.64	0.19	0.00	-0.55	0.55	0.91	low
A_32_P107994	ATG7	1.51	2.07	0.83	0.17	0.02	-0.57	0.57	0.68	low
A_32_P199824	LMOD1	1.55	2.22	0.84	0.12	0.02	-0.67	0.67	0.71	low
A_32_P20997	Transcribed	0.25	0.69	0.15	0.00	0.28	-0.44	0.44	0.10	high
A_32_P35668	666399	1.31	1.71	0.71	0.28	0.02	-0.41	0.41	0.60	low
A_32_P80016	593535	1.59	2.02	0.82	0.26	0.00	-0.42	0.42	0.78	low

Figure S7: The Kullback-Leibler divergence between UGT expression in tumours and healthy samples with high and low MD respectively. Small divergence means more similar distribution in the two populations tested. For three of four UGT probes, the distribution in tumour samples is more similar to the distribution in healthy individuals with high-MD than with low-MD. (The first UGT2B10-probe is A_23_P7342, the probe that is significant in the GLM-analysis, the second is A_24_P521559).

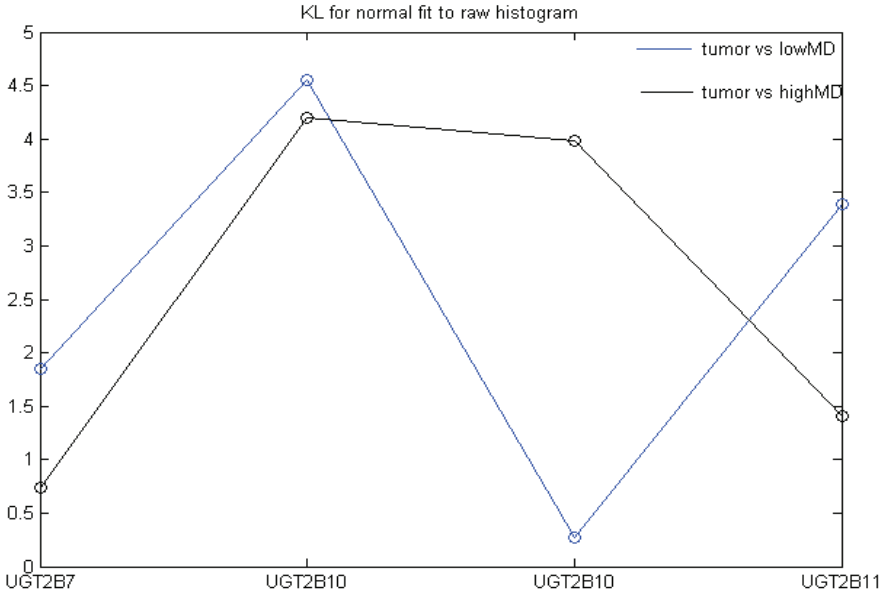


Table S5: Gene expression of UGT2B10 in tumour samples (T) and normal adjacent samples (N) from the same breast in an unpublished dataset. There is no significant difference in mean expression by pair wise t-test.

	A_23_P7342 UGT2B10		A_23_P7342 UGT2B10
CM 1N	9.75	CM 1T	7.52
CM 9N	8.62	CM 9T	8.49
CM 10N	8.77	CM 10T	7.27
CM 11N	9.26	CM 11T	6.65
CM 13N	10.32	CM 13T	7.43
CM 18N	10.69	CM 18T	8.79
CM 19N	9.12	CM 19T	9.11
CM 26N	8.78	CM 26T	7.31
CM 31N	8.00	CM 31T	8.07
CM 32N	7.73	CM 32T	7.23
CM 38N	10.96	CM 38T	7.32
CM 41N	9.07	CM 41T	11.71
CM 46N	8.74	CM 46T	11.21
CM 47N	11.08	CM 47T	8.00
CM 54N	7.80	CM 54T	13.14
CM 56N	7.92	CM 56T	17.48
CMG24N	6.66	CMG24T	13.89
CMG43N	8.24	CMG43T	10.59
CM 44N	8.89	CM 44T	7.75
average	8.97	average	9.42
p-value	0.60	(pair wise t-test)	

Table S6: Range of MD in the breasts of healthy women

max	**77.3133466**
min	**1.28417454**
mean	**28.1668549**
median	**23.5504612**

Supplemental discussion

Gene expression microarray analyses using tissue adjacent to a breast tumour have previously been done [1,2]. The expression profile in these normal samples will be influenced by the neighbouring breast tumour [3]. Breast reduction mammoplasties have also been used in analysis of healthy breast tissue [4]. These samples are generally collected from large breasts with a higher than average proportion of fatty tissue which may also skew the analyses to some extent. Our study analysed a population more representative of the population of women at risk for developing breast cancer, since we have studied normal breast tissue from women with no malignant disease and not undergoing breast reduction mammoplasties.

Reference List

1. Chen DT, Nasir A, Culhane A, Venkataramu C, Fulp W, Rubio R, Wang T, Agrawal D, McCarthy SM, Gruidl M et al.: **Proliferative genes dominate malignancy-risk gene signature in histologically-normal breast tissue.** *Breast Cancer Res Treat* 2009.

2. Yang WT, Lewis MT, Hess K, Wong H, Tsimelzon A, Karadag N, Cairo M, Wei C, Meric-Bernstam F, Brown P et al.: **Decreased TGFbeta signaling and increased COX2 expression in high risk women with increased mammographic breast density.** *Breast Cancer Res Treat* 2009.

3. Showe MK, Vachani A, Kossenkov AV, Yousef M, Nichols C, Nikonova EV, Chang C, Kucharczuk J, Tran B, Wakeam E et al.: **Gene Expression Profiles in Peripheral Blood Mononuclear Cells Can Distinguish Patients with Non-Small Cell Lung Cancer from Patients with Nonmalignant Lung Disease.** *Cancer Res* 2009.

4. Jones C, Mackay A, Grigoriadis A, Cossu A, Reis-Filho JS, Fulford L, Dexter T, Davies S, Bulmer K, Ford E et al.: **Expression profiling of purified normal human luminal and myoepithelial breast cells: identification of novel prognostic markers for breast cancer.** *Cancer Res* 2004, **64:**3037-3045.

Additional File 3 - SAM MD, healthy women, stratified

Healthy women MD 20+

n= 61

Down-regulated in high MD

Gene ID	Gene Name	Score(d)	q-value(%)
A_23_P212968	UGT2B11	-2.7797	0
A_24_P521559	UGT2B10	-2.7043	0
A_24_P180243	UGT2B28	-2.492	0
A_24_P17691	UGT2B17	-2.4912	0
A_23_P7342	UGT2B11	-2.4377	0
A_23_P136671	UGT2B7	-2.3951	0
A_24_P575267	835938	-2.2204	16.580614
A_23_P309739	ESR1	-2.119	29.016075

Healthy women age 50+

n= 43

Down-regulated in high MD

Gene ID	Gene Name	Score(d)	q-value(%)
A_24_P932736	729641	-2.85	0
A_23_P102071	A_23_P102071	-2.80	0
A_24_P131589	CD86	-2.73	0
A_24_P18802	VPS18	-2.68	0
A_23_P144244	810781	-2.64	0
A_23_P427472	FLJ10404	-2.58	0
A_23_P254212	DIAPH2::RPA4	-2.55	0
A_32_P199824	LMOD1	-2.54	0
A_32_P107994	ATG7	-2.52	0
A_24_P111096	PFKFB3	-2.50	0
A_32_P35668	666399	-2.47	0
A_23_P152570	580687	-2.43	0
A_32_P133840	TMCC2	-2.40	5.21
A_24_P375205	MKL2	-2.40	5.21
A_23_P69699	NPY1R	-2.36	5.21
A_24_P177236	CABP7	-2.36	5.21
A_32_P80016	593535	-2.35	5.21
A_24_P693448	ZNF552	-2.35	5.21
A_23_P155463	LRRC2	-2.31	5.21
A_23_P366376	TDGF3	-2.29	5.21
A_23_P66543	PIK3R5	-2.25	5.21
A_24_P640617	Transcribed	-2.25	5.21
A_24_P913847	797019	-2.22	5.21
A_24_P46484	RBM22	-2.21	5.21
A_23_P204277	H2AFJ	-2.16	7.22
A_32_P111996	MGC39584	-2.16	7.22
A_23_P39095	CGB::CGB1	-2.12	8.80
A_23_P119448	SAPS1	-2.11	8.80
A_32_P20997	Transcribed	-2.11	8.80
A_24_P287664	PLCB2	-2.11	8.80
A_32_P77416	554489	-2.11	8.80
A_24_P919640	CD44	-2.10	8.80
A_24_P125894	PPM1F	-2.08	11.04
A_24_P349633	FLJ32679::GOLGA8	-2.07	11.04
A_23_P116694	RPS26	-2.06	11.04
A_23_P436284	OSTbeta	-2.01	13.03
A_24_P315014	825337	-2.01	13.03
A_24_P36890	RAP1GAP	-1.99	14.81
A_23_P44663	SERPINA1	-1.93	16.42
A_32_P149404	537146	-1.93	16.42
A_32_P79313	FLJ45244	-1.92	18.31
A_24_P110601	834483	-1.90	18.31
A_23_P414793	CP	-1.90	18.31
A_23_P38732	CDH2	-1.89	20.40
A_24_P82880	TPM4	-1.89	20.40
A_32_P147241	PKM2	-1.88	20.40
A_32_P168431	RPS26	-1.85	23.46
A_23_P428184	HIST1H2AD	-1.85	23.46
A_24_P719081	786677	-1.83	24.89

Healthy women age <50

n= 30

Down-regulated in high MD

Gene ID	Gene Name	Score(d)	q-value(%)
A_23_P31816	DEFA1	-2.42	108.98
A_24_P945408	A_24_P945408	-2.11	108.98
A_23_P28485	GCA	-2.09	108.98
A_23_P133606	SLC12A2	-1.96	108.98
A_23_P155666	ASAHL	-1.85	108.98
A_24_P521559	UGT2B10	-1.85	108.98
A_23_P57961	PLXNB1	-1.85	108.98
A_23_P251002	A_23_P251002	-1.76	108.98
A_24_P180243	UGT2B28	-1.73	108.98
A_24_P682550	805257	-1.73	108.98
A_24_P926053	EEF1D	-1.73	108.98
A_24_P234732	MXD4	-1.72	108.98
A_23_P7342	UGT2B10	-1.72	108.98
A_23_P218144	LTBP2	-1.68	108.98
A_23_P136671	UGT2B7	-1.67	108.98
A_23_P55616	SLC14A1	-1.67	108.98
A_24_P649357	LOC153561::SMA3	-1.66	108.98
A_24_P105913	660721	-1.64	108.98
A_23_P1833	B3GAT1	-1.62	108.98
A_23_P66481	RTN4RL1	-1.62	108.98
A_24_P942694	C10orf118	-1.62	108.98
A_23_P212968	UGT2B11	-1.61	108.98

Healthy women currently using HT

n=11

Down-regulated in high MD

Gene ID	Gene Name	Score(d)	q-value(%)
A_23_P150979	SBEM	-2.02	0
A_23_P8702	PIP	-1.99	0
A_23_P136671	UGT2B7	-1.77	0
A_23_P393099	TFF3	-1.73	0
A_24_P701582	755742	-1.71	0
A_23_P212968	UGT2B11	-1.70	0
A_24_P180243	UGT2B28	-1.61	14.86

Healthy women ≥ 50 not currently using HT

n= 32

Down-regulated in high MD

Gene ID	Gene Name	Score(d)	q-value(%)
A_23_P39095	CGB::CGB1	-2.31	13.41
A_24_P131589	CD86	-2.24	13.41
A_23_P102071	A_23_P102071	-2.24	13.41
A_32_P199824	LMOD1	-2.16	13.41
A_24_P932736	729641	-2.14	13.41
A_23_P144244	810781	-2.12	13.41
A_24_P640617	Transcribed	-2.10	13.41
A_24_P18802	VPS18	-2.09	13.41
A_23_P366376	TDGF3	-2.07	13.41
A_23_P436284	OSTbeta	-2.07	13.41
A_24_P111096	PFKFB3	-2.06	13.41
A_23_P254212	DIAPH2::RPA4	-2.06	13.41
A_32_P80016	593535	-2.05	13.41
A_23_P152570	580687	-2.04	13.41
A_23_P155463	LRRC2	-2.03	13.41
A_24_P913847	797019	-2.02	13.41
A_23_P66543	PIK3R5	-2.00	13.41
A_24_P919640	CD44	-1.98	13.41
A_32_P133840	TMCC2	-1.97	13.41
A_32_P107994	ATG7	-1.97	13.41
A_32_P149404	537146	-1.89	20.43

Additional file 4 - eQTL

UGT transcripts the expression of which is associated to SNPs in their own or other UGT genes in cis

Probe_ID	Gene exp	SNP_rs	SNP_gene	b1_p
A_23_P41553	Ncaml	rs1828705	UGT2B10	0.01
A_24_P575267	835938	rs1828705	UGT2B10	0.02
A_23_P136671	UGT2B7	rs1828705	UGT2B10	0.04
A_23_P41553	Ncaml	rs941389	UGT2B4	0.05
A_24_P180243	UGT2B28	rs1828705	UGT2B10	0.05
A_24_P180243	UGT2B28	rs2288741	UGT2A1	0.07
A_23_P7342	UGT2B11	rs2288741	UGT2A1	0.08
A_23_P58407	UGT2B15	rs1828705	UGT2B10	0.08
A_24_P180243	UGT2B28	rs4554145	UGT2B4	0.08
A_24_P575267	835938	rs4554145	UGT2B4	0.08
A_24_P575267	835938	rs13139888	UGT2B4	0.08
A_23_P41553	Ncaml	rs4557343	UGT2B4	0.08
A_23_P7342	UGT2B11	rs1560605	UGT2A1	0.09
A_24_P180243	UGT2B28	rs1560605	UGT2A1	0.09
A_23_P212968	UGT2B11	rs1828705	UGT2B10	0.10
A_24_P575267	835938	rs3775782	UGT2A1	0.10
A_24_P180243	UGT2B28	rs10026603	UGT2A1	0.11
A_24_P575267	835938	rs2288741	UGT2A1	0.13
A_24_P180243	UGT2B28	rs1432329	UGT2A1	0.13
A_24_P575267	835938	rs1560605	UGT2A1	0.15
A_24_P180243	UGT2B28	rs3775782	UGT2A1	0.15
A_23_P58407	UGT2B15	rs1454254	UGT2B15	0.16
A_24_P575267	835938	rs1131878	UGT2B4	0.16
A_24_P521559	UGT2B10	rs10026603	UGT2A1	0.16
A_23_P7342	UGT2B11	rs1432329	UGT2A1	0.19
A_24_P575267	835938	rs1432329	UGT2A1	0.20
A_23_P7342	UGT2B11	rs10026603	UGT2A1	0.21
A_24_P180243	UGT2B28	rs13139888	UGT2B4	0.21
A_24_P180243	UGT2B28	rs1131878	UGT2B4	0.21
A_23_P212968	UGT2B11	rs2288741	UGT2A1	0.23
A_23_P212968	UGT2B11	rs1560605	UGT2A1	0.23
A_23_P7342	UGT2B11	rs1828705	UGT2B10	0.23
A_24_P521559	UGT2B10	rs2288741	UGT2A1	0.24
A_24_P180243	UGT2B28	rs4557343	UGT2B4	0.25
A_23_P7342	UGT2B11	rs4557343	UGT2B4	0.25
A_24_P521559	UGT2B10	rs1560605	UGT2A1	0.28
A_23_P7342	UGT2B11	rs3775782	UGT2A1	0.29
A_24_P521559	UGT2B10	rs1828705	UGT2B10	0.29
A_24_P521559	UGT2B10	rs4554145	UGT2B4	0.29
A_23_P7342	UGT2B11	rs4554145	UGT2B4	0.30
A_24_P17691	UGT2B17	rs1828705	UGT2B10	0.31
A_23_P41553	Ncaml	rs3775782	UGT2A1	0.31
A_24_P521559	UGT2B10	rs4235126	UGT2B28	0.33
A_23_P212968	UGT2B11	rs7668258	UGT2B7	0.34
A_23_P212968	UGT2B11	rs4521414	UGT2B7	0.34
A_23_P41553	Ncaml	rs1513559	UGT2B10	0.35
A_23_P212968	UGT2B11	rs10026603	UGT2A1	0.35
A_24_P575267	835938	rs4557343	UGT2B4	0.36
A_23_P212968	UGT2B11	rs7439366	UGT2B7	0.36
A_23_P41553	Ncaml	rs844342	UGT2B10	0.37
A_23_P136671	UGT2B7	rs4554145	UGT2B4	0.38
A_23_P212968	UGT2B11	rs3775782	UGT2A1	0.38
A_23_P136671	UGT2B7	rs10026603	UGT2A1	0.38
A_24_P180243	UGT2B28	rs4694211	UGT2B4	0.40
A_24_P521559	UGT2B10	rs3775782	UGT2A1	0.41
A_24_P521559	UGT2B10	rs1432329	UGT2A1	0.41
A_23_P7342	UGT2B11	rs13139888	UGT2B4	0.41
A_23_P136671	UGT2B7	rs3775782	UGT2A1	0.42
A_23_P136671	UGT2B7	rs2288741	UGT2A1	0.44
A_23_P41553	Ncaml	rs1454254	UGT2B15	0.44
A_23_P41553	Ncaml	rs4554145	UGT2B4	0.44
A_23_P7342	UGT2B11	rs1131878	UGT2B4	0.45
A_24_P575267	835938	rs2045100	UGT2B15	0.45
A_24_P180243	UGT2B28	rs2045100	UGT2B15	0.46
A_23_P212968	UGT2B11	rs1432329	UGT2A1	0.46
A_24_P521559	UGT2B10	rs13139888	UGT2B4	0.47
A_23_P58407	UGT2B15	rs2045100	UGT2B15	0.47
A_24_P521559	UGT2B10	rs1131878	UGT2B4	0.48
A_23_P136671	UGT2B7	rs1560605	UGT2A1	0.48
A_24_P521559	UGT2B10	rs903446	UGT2B4	0.49
A_24_P575267	835938	rs10026603	UGT2A1	0.49
A_23_P41553	Ncaml	rs13139888	UGT2B4	0.50
A_23_P212968	UGT2B11	rs4554145	UGT2B4	0.51
A_23_P212968	UGT2B11	rs2045100	UGT2B15	0.51
A_24_P575267	835938	rs4694211	UGT2B4	0.51
A_23_P58407	UGT2B15	rs1513559	UGT2B10	0.55
A_23_P212968	UGT2B11	rs4557343	UGT2B4	0.57
A_24_P521559	UGT2B10	rs7439366	UGT2B7	0.59
A_23_P58407	UGT2B15	rs7668258	UGT2B7	0.59
A_23_P58407	UGT2B15	rs4521414	UGT2B7	0.59
A_23_P136671	UGT2B7	rs13139888	UGT2B4	0.59
A_23_P136671	UGT2B7	rs4557343	UGT2B4	0.60
A_23_P7342	UGT2B11	rs7668258	UGT2B7	0.60
A_23_P7342	UGT2B11	rs4521414	UGT2B7	0.60
A_23_P136671	UGT2B7	rs2045100	UGT2B15	0.61
A_24_P180243	UGT2B28	rs7439366	UGT2B7	0.62
A_23_P136671	UGT2B7	rs941389	UGT2B4	0.62
A_24_P575267	835938	rs4148279	UGT2A1	0.63
A_24_P17691	UGT2B17	rs1513559	UGT2B10	0.63
A_24_P575267	835938	rs1513559	UGT2B10	0.64
A_23_P136671	UGT2B7	rs1432329	UGT2A1	0.64
A_24_P575267	835938	rs7668258	UGT2B7	0.65
A_24_P575267	835938	rs4521414	UGT2B7	0.65
A_24_P575267	835938	rs4235126	UGT2B28	0.65
A_23_P136671	UGT2B7	rs4694211	UGT2B4	0.66
A_23_P212968	UGT2B11	rs13139888	UGT2B4	0.67
A_23_P136671	UGT2B7	rs1131878	UGT2B4	0.67
A_24_P17691	UGT2B17	rs1454254	UGT2B15	0.68
A_23_P7342	UGT2B11	rs7439366	UGT2B7	0.68
A_24_P17691	UGT2B17	rs844342	UGT2B10	0.69
A_23_P7342	UGT2B11	rs4148279	UGT2A1	0.69
A_24_P521559	UGT2B10	rs4694211	UGT2B4	0.70
A_23_P7342	UGT2B11	rs844342	UGT2B10	0.70
A_24_P521559	UGT2B10	rs7668258	UGT2B7	0.71
A_24_P521559	UGT2B10	rs4521414	UGT2B7	0.71
A_24_P521559	UGT2B10	rs844342	UGT2B10	0.72
A_23_P212968	UGT2B11	rs1131878	UGT2B4	0.73
A_23_P41553	Ncaml	rs4235126	UGT2B28	0.73
A_24_P180243	UGT2B28	rs4235126	UGT2B28	0.73
A_24_P180243	UGT2B28	rs844342	UGT2B10	0.74
A_23_P7342	UGT2B11	rs903446	UGT2B4	0.74
A_24_P521559	UGT2B10	rs941389	UGT2B4	0.75
A_23_P136671	UGT2B7	rs4235126	UGT2B28	0.75
A_24_P521559	UGT2B10	rs2045100	UGT2B15	0.76
A_24_P575267	835938	rs7439366	UGT2B7	0.77
A_23_P7342	UGT2B11	rs941389	UGT2B4	0.77
A_24_P575267	835938	rs844342	UGT2B10	0.78
A_24_P180243	UGT2B28	rs7668258	UGT2B7	0.78
A_24_P180243	UGT2B28	rs4521414	UGT2B7	0.78
A_23_P136671	UGT2B7	rs4148279	UGT2A1	0.78
A_23_P7342	UGT2B11	rs4235126	UGT2B28	0.79
A_23_P212968	UGT2B11	rs4694211	UGT2B4	0.79
A_23_P41553	Ncaml	rs1131878	UGT2B4	0.79
A_24_P575267	835938	rs1454254	UGT2B15	0.80
A_23_P7342	UGT2B11	rs1513559	UGT2B10	0.80
A_23_P58407	UGT2B15	rs7439366	UGT2B7	0.80
A_23_P41553	Ncaml	rs7439366	UGT2B7	0.82
A_23_P41553	Ncaml	rs7668258	UGT2B7	0.83
A_23_P41553	Ncaml	rs4521414	UGT2B7	0.83
A_24_P521559	UGT2B10	rs4557343	UGT2B4	0.83
A_23_P58407	UGT2B15	rs844342	UGT2B10	0.83
A_23_P41553	Ncaml	rs4694211	UGT2B4	0.83
A_23_P7342	UGT2B11	rs2045100	UGT2B15	0.84
A_23_P212968	UGT2B11	rs903446	UGT2B4	0.84

Probe_ID	Gene exp	SNP_rs	SNP_gene	b1_p
A_23_P212968	UGT2B11	rs4235126	UGT2B28	0.84
A_23_P212968	UGT2B11	rs844342	UGT2B10	0.84
A_24_P180243	UGT2B28	rs1454254	UGT2B15	0.85
A_24_P180243	UGT2B28	rs903446	UGT2B4	0.87
A_23_P212968	UGT2B11	rs1513559	UGT2B10	0.87
A_23_P136671	UGT2B7	rs844342	UGT2B10	0.88
A_24_P180243	UGT2B28	rs4148279	UGT2A1	0.90
A_24_P521559	UGT2B10	rs4148279	UGT2A1	0.90
A_24_P521559	UGT2B10	rs1513559	UGT2B10	0.90
A_23_P41553	Ncaml	rs903446	UGT2B4	0.90
A_23_P136671	UGT2B7	rs7668258	UGT2B7	0.90
A_23_P136671	UGT2B7	rs4521414	UGT2B7	0.90
A_23_P212968	UGT2B11	rs941389	UGT2B4	0.91
A_24_P521559	UGT2B10	rs1454254	UGT2B15	0.93
A_23_P7342	UGT2B11	rs1454254	UGT2B15	0.93
A_23_P212968	UGT2B11	rs1454254	UGT2B15	0.94
A_23_P41553	Ncaml	rs2045100	UGT2B15	0.95
A_24_P17691	UGT2B17	rs2045100	UGT2B15	0.95
A_24_P180243	UGT2B28	rs1513559	UGT2B10	0.96
A_23_P136671	UGT2B7	rs903446	UGT2B4	0.96
A_24_P575267	835938	rs941389	UGT2B4	0.97
A_23_P136671	UGT2B7	rs1454254	UGT2B15	0.98
A_23_P7342	UGT2B11	rs4694211	UGT2B4	0.98
A_23_P212968	UGT2B11	rs4148279	UGT2A1	0.98
A_23_P41553	Ncaml	rs2288741	UGT2A1	0.98
A_23_P136671	UGT2B7	rs1513559	UGT2B10	0.99
A_23_P136671	UGT2B7	rs7439366	UGT2B7	0.99
A_24_P180243	UGT2B28	rs941389	UGT2B4	1.00
A_23_P362694	C4orf7	rs903446	UGT2B4	0.01
A_23_P41365	SMR3A	rs941389	UGT2B4	0.06
A_23_P362694	C4orf7	rs7439366	UGT2B7	0.10
A_23_P362694	C4orf7	rs4148279	UGT2A1	0.10
A_23_P362694	C4orf7	rs7668258	UGT2B7	0.14
A_23_P362694	C4orf7	rs4521414	UGT2B7	0.14
A_23_P110234	CSN1S1	rs3775782	UGT2A1	0.18
A_23_P110234	CSN1S1	rs13139888	UGT2B4	0.18
A_23_P110234	CSN1S1	rs1131878	UGT2B4	0.19
A_23_P362694	C4orf7	rs1131878	UGT2B4	0.22
A_23_P362694	C4orf7	rs4554145	UGT2B4	0.23
A_23_P110234	CSN1S1	rs4554145	UGT2B4	0.24
A_23_P41365	SMR3A	rs3775782	UGT2A1	0.27
A_23_P362694	C4orf7	rs4235126	UGT2B28	0.33
A_23_P110234	CSN1S1	rs4694211	UGT2B4	0.34
A_23_P41365	SMR3A	rs4557343	UGT2B4	0.40
A_23_P362694	C4orf7	rs4694211	UGT2B4	0.43
A_23_P110234	CSN1S1	rs1432329	UGT2A1	0.49
A_23_P362694	C4orf7	rs3775782	UGT2A1	0.52
A_23_P41365	SMR3A	rs13139888	UGT2B4	0.52
A_23_P362694	C4orf7	rs2288741	UGT2A1	0.58
A_23_P110234	CSN1S1	rs4557343	UGT2B4	0.58
A_23_P41365	SMR3A	rs903446	UGT2B4	0.61
A_23_P41365	SMR3A	rs4554145	UGT2B4	0.64
A_23_P110234	CSN1S1	rs4235126	UGT2B28	0.68
A_23_P41365	SMR3A	rs4148279	UGT2A1	0.70
A_23_P110234	CSN1S1	rs941389	UGT2B4	0.75
A_23_P362694	C4orf7	rs1432329	UGT2A1	0.75
A_23_P362694	C4orf7	rs941389	UGT2B4	0.78
A_23_P110234	CSN1S1	rs1560605	UGT2A1	0.78
A_23_P110234	CSN1S1	rs2288741	UGT2A1	0.79
A_23_P41365	SMR3A	rs1131878	UGT2B4	0.81
A_23_P110234	CSN1S1	rs844342	UGT2B10	0.81
A_23_P362694	C4orf7	rs10026603	UGT2A1	0.81
A_23_P41365	SMR3A	rs4694211	UGT2B4	0.84
A_23_P110234	CSN1S1	rs7439366	UGT2B7	0.85
A_23_P41365	SMR3A	rs1432329	UGT2A1	0.85
A_23_P362694	C4orf7	rs1560605	UGT2A1	0.85
A_23_P110234	CSN1S1	rs7668258	UGT2B7	0.86
A_23_P110234	CSN1S1	rs4521414	UGT2B7	0.86
A_23_P110234	CSN1S1	rs10026603	UGT2A1	0.88
A_23_P362694	C4orf7	rs4557343	UGT2B4	0.89

Probe_ID	Gene exp	SNP_rs	SNP_gene	b1_p
A_23_P41365	SMR3A	rs2288741	UGT2A1	0.90
A_23_P41365	SMR3A	rs10026603	UGT2A1	0.91
A_23_P362694	C4orf7	rs13139888	UGT2B4	0.92
A_23_P110234	CSN1S1	rs903446	UGT2B4	0.92
A_23_P41365	SMR3A	rs1560605	UGT2A1	0.94
A_23_P41365	SMR3A	rs4235126	UGT2B28	0.96
A_23_P110234	CSN1S1	rs4148279	UGT2A1	0.98

Paper III

Serum estradiol levels associated with specific gene expression patterns in normal breast tissue and in breast carcinomas

Serum estradiol levels associated with specific gene expression patterns in normal breast tissue and in breast carcinomas

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Conflict of interest:

The authors claim no conflict of interest.

Running title:
Gene expression associated with serum estradiol in normal and malignant breast tissue

Keywords:
Serum estradiol, *SCGB3A1, HIN1, TLN2, PTGS1, COX1, AREG, GREB1, TFF*, normal breast tissue, gene expression.

Abstract

Introduction

High serum levels of estradiol are associated with increased risk of breast cancer. Little is known about the gene expression in normal breast tissue in relation to levels of circulating serum estradiol.

Methods

We compared whole genome expression data of breast tissue samples with serum hormone levels using data from 79 healthy women and 64 breast cancer patients. Significance analysis of microarrays (SAM) was used to identify differentially expressed genes and multivariate linear regression was used to identify independent associations.

Results

Six genes (*SCGB3A1, RSPO1, TLN2, SLITRK4, DCLK1, PTGS1*) were found differentially expressed according to serum estradiol levels (FDR=0). Three of these were independent predictors of estradiol levels in a multivariate model: *SCGB3A1* (*HIN1*) and *TLN2* were up-regulated and *PTGS1* (*COX1*) was down-regulated in samples form women with high serum estradiol. *SCGB3A1* is a suggested tumor suppressor gene that inhibits cell growth and invasion and is methylated and down-regulated in many epithelial cancers. PTGS1 induces prostaglandin E2 (PGE2) production which in turn stimulates aromatase expression and hence increases the local production of estradiol. Serum estradiol, but none of the differentially expressed genes were significantly associated with mammographic density, another strong breast cancer risk factor. In breast carcinomas, expression of *GREB1* and *AREG* was associated with serum estradiol in all cancers and in the subgroup of estrogen receptor positive cases.

Conclusion

We have identified genes associated with serum estradiol levels in normal breast tissue and in breast carcinomas. This is the firs report studying such associations in normal breast tissue in humans. Serum estradiol, but none of the differentially expressed genes, was found to be an independent predictor of mammographic density.

Introduction

Influence of estradiol on breast development [1], the menopausal transition [2] and on the breast epithelial cells [3] is widely studied. However, little is known about the effect of serum estradiol on gene expression in the normal breast tissue. For post-menopausal women, high serum estradiol levels are associated with increased risk of breast cancer [4-6]. The results are less conclusive for premenopausal women, but epidemiologic evidence indicates an increased risk from higher exposure to female hormones [7].

In estrogen receptor (ER) positive breast carcinomas, the proliferating tumor cells express ER while in normal breast tissue the proliferating epithelial cells are ER negative (ER-) [8,9]. Both normal and malignant breast epithelial cells are influenced by estradiol but through different mechanisms. In the lack of ER, normal breast epithelial cells receive proliferating paracrine signals from ER+ fibroblasts [3]. The importance of estrogen stimuli in the proliferation of ER+ breast cancer cells is evident from the effect of anti-estrogen treatment. Previously, several studies have identified genes whose expression is regulated by estradiol in breast cancer cell lines. Recently, a study reported an association between serum levels of estradiol and gene expression of TFF1, GREB1, PDZK1 and PGR in ER+ breast carcinomas [10]. Functional studies on breast cancer cell lines have described that estradiol induces expression of *c-fos* [11] and that exposure to physiologic doses of estradiol is necessary for malignant transformation [12]. Intratumoral levels of estrogens have also been measured and were found correlated with tumor gene expression of estradiol-metabolizing enzymes and ESR1 [13] and of proliferation markers [14]. A recent study did, however, conclude that the intratumoral estradiol levels were mainly determined by its binding to ER (associated with ESR1-expression). The intratumoral estradiol levels were not found to be associated with local estradiol production [15]. Serum estradiol levels were found to be associated with local estradiol levels in normal breast tissue of breast cancer patients in a recent study [16]. This strengthens the hypothesis that serum estradiol levels influence the gene expression in breast tissue.

Wilson and colleagues studied the effect of estradiol on normal human breast tissue transplanted into athymic nude mice. They identified a list of genes associated with estradiol treatment, including TFF1, AREG, SCGB2A2, GREB1 and GATA3. The normal tissues used in the xenografts were from breasts with benign breast disease and from mammoplasty reductions [17].

Studies describing associations between serum estradiol levels and gene expression of normal human breast tissue in its natural milieu are lacking. Knowledge about gene expression changes associated with high serum estradiol may reveal biological mechanisms underlying the increased risk for both elevated mammographic density and for developing breast cancer as seen in women with high estradiol levels. We have identified genes differentially expressed between normal breast tissue samples according to serum estradiol levels. Several genes identified in previous studies using normal breast tissue or breast carcinomas are confirmed, but additional genes were identified making important contributions to our previous knowledge.

Materials and methods

Subjects

Two cohorts of women were recruited to the study from different breast diagnostic centers in Norway in the period 2002-2007 as described previously [18]. Exclusion criteria were pregnancy and use of anticoagulant therapy. The first cohort consisted of women referred to the breast diagnostic centers who were cancer-free after further evaluation. Breast biopsies were taken from an area with some mammographic density in the breast contralateral to any suspect lesion. The second cohort consisted of women who were diagnosed with breast cancer. For this cohort, study biopsies were taken from the breast carcinoma after the diagnostic biopsies were obtained. Fourteen gauge needles were used for the biopsies and sampling was guided by ultrasound. The biopsies were either soaked in RNAlater (Ambion, Austin, TX) and sent to the Oslo University Hospital, Radiumhospitalet, before storage at -20°C or directly snap-frozen in liquid nitrogen and stored at -80°C.

All women provided information about height, weight, parity, hormone therapy use and family history of breast cancer and provided a signed informed consent. The study was approved by the regional ethical committee (IRB approval no S-02036). In total, 120 healthy women with no malignant disease were recruited in the first cohort. These are in the following referred to as 'healthy women'. In the second cohort, 66 women with a newly diagnosed breast cancer were recruited.

Three additional datasets were used to explore the regulation of identified genes in breast cancer. One unpublished dataset from the Akershus University Hospital (AHUS), Norway, included normal breast tissue from 42 reduction mammoplasties and both tumor and normal adjacent tissue from 48 breast cancer patients (referred to as the AHUS dataset). Another unpublished dataset from University of North Carolina (UNC), USA, included breast cancer and adjacent normal breast tissue from 55 breast cancer patients (referred to as the UNC dataset). The third dataset is previously published and consists of biopsies from 31 pure ductal carcinoma in situ (DCIS), 36 pure invasive breast cancers and 42 tumours with mixed histology, both DCIS and invasive [19].

Serum hormone analysis

Serum hormone levels (LH, FSH, prolactin, estradiol, progesterone, SHBG and testosterone) were measured with electrochemiluminescence immunoassays (*ECLIA*) on a Roche Modular E instrument (Roche, Basel, Switzerland) by Department of Medical Biochemistry, Oslo University Hospital, Rikshospitalet. The menopausal status was determined based on serum levels of hormones, age and hormone use. The criteria used can be found in Supplementary Table S1. Biochemically perimenopausal women or women with uncertain menopausal status were excluded from analyses stratified on menopause. These hormone assays are tested through an external quality assessment scheme, Labquality, and the laboratory is accredited according to ISO–ES 17025. Serum estradiol values are given as picograms per milliliter (pg/ml).

Gene expression analysis

RNA extraction and hybridization were performed as previously described [18]. Briefly, RNeasy Mini Protocol (Qiagen, Valencia, CA) was used for RNA extraction. Forty samples (38 from healthy women) were excluded from further analysis due to low RNA amount (<10ng) or poor RNA quality (measured by Agilent Bioanalyzer, Agilent Technologies, Palo Alto, CA). Agilent Low RNA input Fluorescent Linear Amplification Kit Protocol was used for amplification and labelling with Cy5 (Amersham Biosciences, Little Chalfont, England) for sample RNA and Cy3 (Amersham Biosciences, Little Chalfont, England) for the reference (Universal Human total RNA (Stratagene, La Jolla, CA)). Labelled RNA was hybridized onto Agilent Human Whole Genome Oligo Microarrays (G4110A) (Agilent Technologies, Santa Clara, CA). Three arrays were excluded due to poor quality leaving data from 79 healthy women and 64 breast cancer patients.

The scanned data was processed in Feature Extraction 9.1.3.1 (Agilent Technologies, Santa Clara, CA). Locally weighted scatterplot smoothing (lowess) was used to normalize the data. The normalized and log2-transformed data was stored in the Stanford Microarray Database (SMD)[20] and retrieved for further analysis. Gene filtering excluded probes with ≥20% missing values and probes with less than three arrays being at least 1.6 standard deviation away from the mean. This reduced the dataset from 40791 probes to 9767 for the healthy women and to 10153 for the breast cancer patients. Missing values were imputed in R using the method impute.knn in the library impute [21].

Mammographic density

Mammographic density was estimated from digitized craniocaudal mammograms as previously described [18] using the University of Southern California Madena assessment method [22]. First, the total breast area was outlined using a computerized tool and the area was represented as number of pixels. One of the co-authors, GU, identified a region of interest that incorporated all dense areas of density excluding those representing the pectoralis muscle and scanning artifacts. All densities above a certain threshold were tinted yellow, and the tinted pixles converted to cm^2 representing the absolute density and was available for 108 of 120 healthy women. Percent mammographic density is calculated as the absolute density divided by the total breast area and was available for 114 of 120 healthy women. Test-retest reliability was 0.99 for absolute density.

Statistical Analysis

Quantitative significance Analysis of Microarrays (SAM) [23,24] was used for analysis of differentially expressed genes, by the library samr in R 2.12.0. Serum estradiol (nmol/L) was used as dependent variable. The distribution of serum levels is skewed and therefore the non-parametric Wilcoxon test-statistic was used. Probes with an FDR<50% were included for gene ontology analyses.

DAVID Bioinformatics Resources 2008 from the National Institute of Allergy and Infectious Diseases, NIH [25] was used for gene ontology analysis. Functional annotation clustering was applied and the following annotation categories were selected: biological processes, molecular function, cellular compartment and KEGG pathways. We included

annotation terms with a p-value (FDR-corrected) of <0.01 containing between 5 and 500 genes.

For multivariate analysis, linear regression was fitted in R 2.12.0 to identify independent associations. Stepwise selection was performed to determine which variables had an independent contribution to the response variable. In the first step, all variables were included in the model. The variable with the highest p-value was rejected from the model in each step, before the model was refitted. This was repeated until all variables in the model had a p-value smaller than 0.05.

Linear regression was used to determine the independent association between serum estradiol and the differentially expressed genes in healthy women. Age, menopause and current hormone use were included in the model and forced to stay throughout the stepwise selection to correct for confounding by these factors. Linear regression was also fitted in two analyses with mammographic density in healthy women as a dependent variable. In one set of analyses serum hormone levels were included as the independent covariates, and in the other analysis, variables representing gene expression associated with serum estradiol were included as covariates. Epidemiologic covariates, such as age, BMI, parity and use of hormone therapy were included in the mammographic density analyses and forced to stay throughout the stepwise selection to control for potential confounding by these factors.

Tumor subtypes were calculated using the intrinsic subtypes published by Sørlie et al in 2001 [26]. The total gene set was filtered for the intrinsic genes. The correlation between gene expression profiles for the intrinsic genes for each sample with each subtype was calculated. Each sample was assigned to the subtype with which it had the highest correlation. Samples with all correlations <0.1 were not assigned to any subtype. Two-sided t-tests were used to check for difference in expression for single genes between two categories of variables (eg: pre- and postmenopausal).

Results

Gene expression in normal breast tissue according to serum estradiol levels

Genes differentially expressed in normal breast tissue from healthy women according to serum estradiol levels with FDR=0 are listed in Table 1. The gene ontology terms *extracellular region* and *skeletal system development* were significantly enriched in the top 80 up-regulated genes (FDR<50%). There were no significant gene ontology terms enriched in the down-regulated genes with FDR<50 (n=8), although *response to steroid hormone stimulus* was the most enriched term with three observed genes (*PTGS1, ESR1* and *GATA3*).

The genes differentially expressed in normal breast tissue according to serum estradiol with an FDR=0 (from Table 1) were tested for differential expression between breast cancer tissue and normal breast tissue from healthy women. All six genes were differentially expressed between carcinomas and normal tissue. Interestingly, the expression in breast carcinomas was similar to that in normal tissue from women with lower levels of circulating estradiol and opposite to that found in normal samples from women with higher levels of serum estradiol (Table 1). Comparing the expression of these genes in normal breast tissue with the expression in ER+ and ER- carcinomas respectively revealed similar results (Table 1).

In tumors, *SCGB3A1* tended to be expressed at a lower level in basal-like tumors compared with all other tumors or compared with luminal A tumors, but this did not reach statistical significance (both p-values=0.2). However in two other datasets (AHUS and UNC), *SCGB3A1* was expressed at significantly lower levels in basal-like tumors compared with all other subtypes (p=0.04 and 0.003 respectively). There was no consistent significant difference in *SCGB3A1* expression in ER+ and ER- tumors.

Of the six genes differentially expressed according to serum estradiol in normal breast tissue, three were differentially expressed between DCIS and early invasive breast carcinomas based on a previously published dataset [19](Table 1). *SCGB3A1* was down-regulated in invasive compared with DCIS, whereas *TLN2* and *PTGS1* were up-regulated in invasive compared with DCIS.

A linear regression was fitted with all differentially expressed genes as covariates and controlling for age, menopause and current hormone therapy use. After leave-one-out elimination of insignificant covariates, *SCGB3A1, TLN2* and *PTGS1* were still significant (Table 2).

Serum estradiol related to mammographic density in healthy women

Regression analysis in postmenopausal women showed that serum estradiol was independently associated with both absolute and percent mammographic density when controlling for age, BMI and current use of hormone therapy (Table 3). None of the genes differentially expressed in normal breast tissue according to serum estradiol levels were independently associated with mammographic density (data not shown).

Gene expression in breast carcinomas according to serum estradiol levels
In breast carcinomas, quantitative SAM revealed two genes, *AREG* and *GREB1*, as differentially expressed according to serum estradiol levels with FDR=0 (Table 4). Both genes were up-regulated in samples from women with high serum estradiol (estradiol was used as a continuous response variable in the analysis). Of 16 probes up-regulated in samples from women with high serum estradiol, there were three probes for *TFF3* and one for *TFF1*, although these did not reach statistical significance (Table 4). No genes were significantly down-regulated. In ER+ samples (n=53), we also found *AREG* and *GREB1* up-regulated in samples from women with high serum estradiol (FDR=0), but the *TFF*-genes were not up-regulated. Among the ER- samples (n=8) there was very little variation in serum estradiol levels and a search for genes differentially expressed according to serum estradiol is not feasible.

Looking at normal breast tissue from healthy women, both *AREG* and *GREB1* are up-regulated in samples from women with high estradiol levels without reaching significance. Neither *AREG* nor *GREB1* are differentially expressed between normal breast tissue and breast carcinomas. All the probes for *TFF*-genes are, however, significantly down-regulated in normal breast tissue compared with breast carcinomas (Supplementary Table 3).

Discussion

Gene expression in normal breast tissue according to serum estradiol levels

We have identified genes differentially expressed according to serum estradiol in normal breast tissue of healthy women.

The genes up-regulated in normal breast tissue under influence of high serum estradiol are enriched for the gene ontology terms *extracellular matrix* and *skeletal system development*. Both ER isoforms α and β are expressed in the stromal cells [27]. The proliferating epithelial cells are not found to be ERα+ [8] and most often negative to both ER isoforms [9]. In normal breast tissue, the estrogen-induced epithelial proliferation is, at least partly, caused by paracrine signals from ER+ fibroblasts [3]. The enrichment of gene ontology terms related to extracellular matrix may be linked to the effect of estradiol on the ER+ stromal cells.

Three genes were independently associated with serum estradiol levels in normal breast tissue in a linear regression model after controlling for age, menopause and current hormone therapy. The two genes *SCBG3A1* (*HIN1*) and *TLN2* were positively associated with serum estradiol and *PTGS1* (*COX1*) negatively.

SCBG3A1 is a secretoglobin transcribed in luminal, but not in myoepithelial breast cells and is secreted from the cell [28]. The protein is a tumor suppressor and inhibits cell growth, migration and invasion acting through the AKT-pathway. SCBG3A1 inhibits Akt-phosphorylation, which reduces the Akt-funtion in promoting cell cycle progression (transition from the G1 to the S-phase) and preventing apoptosis (through inhibition of the TGFβ-pathway) [29] (Figure 1).

The *SCBG3A1* promoter was found to be hypermethylated with down-regulated expression of the gene in breast carcinomas compared with normal breast tissue, where it is referred to as “high in normal 1” (HIN1)[30,31]. Interestingly, the gene is not methylated in BRCA-mutated and BRCA-like breast cancer [32]. Methylation of the gene is suggested to be an early event in non-BRCA-associated breast cancer [33].

We found *SCBG3A1* down-regulated in basal-like cancers compared to other subtypes. At first glance, this may seem contradictory to the observation that the gene is not methylated in BRCA-like breast cancers. However, Krop and colleagues found that the gene is expressed in luminal epithelial cell lines, but not in myoepithelial cell lines. The reduced expression seen in basal-like cancer could be due to a myoepithelial phenotype arising from a myoepithelial cell of origin or from phenotypic changes acquired during carcinogensis. This could also be linked to the lack of methylation in BRCA-associated breast cancers, which are often basal-like. An a priori low gene expression would make methylation unnecessary. The increased Akt-activity seen in basal-like cancers[34] is consistent with the low levels of SCBG3A1 expression observed in the basal-like cancers in this study leading to increased Akt-phosphorylation and thereby Akt-activity.

PTGS1 (prostaglandin-endoperoxide synthase 1) is synonymous with cyclooxygenase 1 (*COX1*) and codes for an enzyme important in prostaglandin production. Studies of normal human adiopocytes have shown that the enzyme induces production of prostaglandin E2 (PGE2) which in turn increases the expression of aromatase (*CYP19A1*) [35]. Aromatase is the enzyme responsible for the last step in the conversion of

androgens to estrogens in adipose tissue. Hence, the expression of *PTGS1* may lead to an increased production of estradiol locally (Figure 2). In normal breast tissue, we observed that the expression of *PTGS1* was lower in samples from women with higher levels of serum estradiol. This may be due to negative feedback. High systemic levels of estradiol make local production unnecessary and PTGS1-induced aromatase production is abolished.

The up-regulation of *PTGS1* in breast carcinomas compared to normal tissue is expected from current knowledge. Several studies have suggested that *PTGS1* has a carcinogenic role in different epithelial cancers [36-40]. The gene has also previously been found over-expressed in tumors compared with tumor adjacent normal tissue [41].

Talin 2 (*TLN2*) is less known and less studied than Talin 1 (*TLN1*). Both talins are believed to connect integrins to the actin cytoskeleton and are involved in integrin-associated cell adhesion [42,43]. *TLN2* is located on chromosome 15q15-21, close to *CYP19A1* coding for aromatase. A study on aromatase-excess syndrome found that certain minor chromosomal rearrangements may cause cryptic transcription of the *CYP19A1* gene through the *TLN2*-promoter [44]. We found that *TLN2* was up-regulated in breasts of healthy women with high levels of serum estradiol. This could indicate an activation of cell adhesion. This gene was the only gene significantly up-regulated according to serum estradiol in normal breast tissue of premenopausal women. The down-regulation observed in breast cancers compared with normal breast tissue indicates a loss of cell adhesion. The expression of the gene is lower in DCIS than in invasive carcinomas, which is contrary to expected, but the data set is small.

A previous study report on the gene expression in normal human breast tissue transplanted into two groups of athymic mice treated with different levels of estradiol [17]. Neither *SCGB3A1*, *TLN2* nor *PTGS1* was significantly differentially expressed in their study. They did, however, identify many of the genes found to be significantly differentially expressed according to serum estradiol in breast carcinomas in the current study, such as *AREG* (amphiregulin), *GREB1 (*growth regulation by estrogen in breast cancer 1), *TFF1* (Trefoil factor 1) and *TFF3* (Trefoil factor 3). Going back to our normal samples, we see that several of their genes (including *AREG*, *GREB1*, *TFF1* and *TFF3*, *GATA3* and two *SERPIN*-genes) are differentially expressed in our normal breast tissue, but did not reach statistical significance (Supplementary table S3).

The differences observed between our study and that of Wilson and colleagues may be due to chance and due to the presence of different residual confounding in the two studies. Wilson and colleagues studied the effects of estradiol treatment, which may act differently upon the breast tissue than endogenous estradiol. Normal human breast tissue transplanted into mice may react differently to varying levels of estradiol than it does in its natural milieu in humans. The genes that were significant in the Wilson-study and differentially expressed but not significant in our study (eg: *AREG*, *GREB1*, *TFF1*, *TFF3* and *GATA3*) may be associated with serum estradiol levels in normal tissues as well as in tumor tissues where we and others have observed significant associations. Our study is the first study to identify the expression of *SCGB3A1*, *TLN2* and *PTGS1* in normal breast tissue to be significantly associated with serum estradiol levels. These findings are biologically reasonable and may have been missed in previous studies due to lack of representative study material.

Serum estradiol associated with mammographic density in healthy women

Serum estradiol levels were independently associated with mammographic density controlling for age, BMI and current use of hormone therapy, and the magnitude of the association was substantial (Table 3). The high beta-value in the regression equation implies a large magnitude of impact which supports the hypothesis that high serum estradiol levels increases mammographic density with both statistical and biological significance.

Gene expression in breast carcinomas according to serum estradiol levels

The expression of genes found to be differentially expressed in normal breast tissue according to serum estradiol levels was examined in breast carcinomas. We found that the expression was all opposite of that in normal breast tissue from women with high serum estrogen (Table 1). This may be due to lack of negative feedback of growth regulation in breast tumors. In breast cancer cell lines, estrogen induced up-regulation of positive proliferation regulators and down-regulation of anti-proliferative and pro-apoptotic genes, resulting in a net positive proliferative drive [45]. This is in line with our findings. In normal breast tissue from women with high serum estradiol, *SCGB3A1*, which regulate proliferation negatively, and *TLN2*, which prevents invasion, are up-regulated. *PTGS1*, which induce local production of estradiol-stimulated proliferation, is down-regulated. All three genes are expressed to maintain control and regulation of the epithelial cells. In breast cancers the expression of these genes favors growth, migration and proliferation. This supports the hypothesis that high serum estradiol increases the proliferative pressure in normal breasts, which leads to an activation of mechanisms counter-acting this proliferative pressure. In carcinomas, growth regulation is lost, and these hormone-related growth-promoting mechanisms are turned on simultaneously.

Interestingly, both *AREG* and *GREB1* were up-regulated in ER+ breast carcinomas of younger (<45 years) compared with older (>70 years) women in a previous publication. The increased expression of these genes was proposed as a mechanism responsible for the observed increase in proliferation seen in the tumors of younger compared with older women [46]

The genes differentially expressed according to serum estradiol levels in tumors confirmed many of the findings from the Dunbier-study of ER+ tumors [10]. The previously published list of genes positively correlated with serum estradiol included three TFF-genes and *GREB1*. These genes were also found significant in the analysis of all tumors in this study, although *TFF1* and *TFF3* did not reach statistical significance (Table 4). In addition to the previously published genes, we identified the gene *AREG*, an EGFR-ligand essential for breast development, as up-regulated in tumors from patients with high serum estradiol.

GREB1 is previously found to be an important estrogen-induced stimulator of growth in ER+ breast cancer cell lines[47]. AREG binds to and stimulates EGFR and hence epithelial cell growth. The up-regulation of these two genes in breast carcinomas of women with high estradiol levels may indicate a loss of regulation of growth associated with cancer development. This corresponds well with the interpretation our findings in normal breast tissue referred above and confirms the results indicated by the cell line studies by Frasor and colleagues [45]. These two genes are not differentially expressed

between normal breast tissue and breast cancers. Both are, however, higher expressed in ER+ than ER- breast carcinomas.

Overall strengths and limitations of the study

The currently used method for detection of serum estradiol has a limited sensitivity in the lower serum levels often seen in postmenopausal women. Despite the limited sample size we found several biologically plausible associations. However, due to limited power, there may be other associations that we could not reveal. We have included women with and without hormone therapy in the study. There may be differences in action between endogenous and exogenous estradiol that will not be revealed in this study.

One important strength of this study is the unique material with normal human tissue in its natural mileu, not influenced by an adjacent tumor[48-50] or by an adipose-dominated biology that may bias the study of reduction mammoplasties.

Conclusion

In conclusion we report a list of genes whose gene expression is associated with serum estradiol levels. This list includes genes with known relation to estradiol-signaling, mammary proliferation and breast carcinogenesis. All these genes were expressed differently in tumor and normal breast tissue. The gene expression in tumors resembled that in normal breast tissue from women with low serum estradiol. Associations between serum estradiol and the expression in breast carcinomas confirmed previous findings and revealed new associations. The comparison of results between normal breast tissue from healthy women and breast carcinomas indicate the difference in biological impact of estradiol in normal and cancerous breast tissue.

Abbreviations

ER: Estrogen receptor. *SCGB3A1*: Secretoglobin 3A1, *HIN1*: High in normal 1, *TLN2*: Talin 2. *PTGS1*: prostaglandin-endoperoxide synthase 1. *COX1*: cyclooxygenase 1. *AREG*: Amphiregulin. *GREB1*: Growth regulation by estrogen in breast cancer 1. *TFF*: Trefoil factor. *GATA3*: GATA binding protein 3. MDG: Mammographic density and genetics. AHUS: Akershus University Hospital. UNC: University of North Carolina. MCF-7: Michigan Cancer Foundation – 7.

Competing interests

The authors declared no competing interests.

Authors' contributions

The trial was designed by ALBD, ÅH, GU, MMH and VNK. ALBD and ÅH ensured funding. MMH, IKB, MR, MAT and VDH assisted in data collection. TB was responsible for serum hormone analyses. VDH and TL contributed to the laboratory work. GU estimated mammographic density. VDH did statistical analyses of the data. ÅH, ALBD and VDH interpreted the results and wrote the paper. All authors were involved in reviewing the report. No medical writers were involved in this paper.

Acknowledgments

This study was funded primarily by The Research Council of Norway and South-Eastern Norway Regional Health Authority. We thank all the women who participated in the study and all the personnel in the hospitals who made the inclusion of these women possible, in particular the responsible radiologists: Jan Ole Frantzen, Linda Romundstad, Dina Navjord, Einar Vigeland, Rolf O Næss and Else Berit Velken. We would also like to thank Lars Ottestad for help in the initiation of the project and Hilde Johnsen and Caroline Jevanord Frøyland for lab assistance.

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Table 1 Genes significantly differentially expressed in normal breast tissue of healthy women according to serum estradiol. A) Q-values and regulation of gene expression from quantitative SAM analysis of gene expression according to serum estradiol. B) Significance testing of difference in gene expression of the genes identified in A) in different sample cohorts.

	Gene Name	*SCGB3A1*	*SLITRK4*	*TLN2*	*DCLK1*	*RSPO1*	*PTGS1*
A	Chromosomal location of the gene	5q35.3	Xq27.3	15q15-q21	13q13	1p34.3	9q32-q33.3
	q-value (%) SAM [1]	0	0	0	0	0	0
	Gene expression in high s-est[2] (compared with low s-est)	up	up	up	up	up	down
B	BC[3] vs normal breast tissue (p-value)[4]	5.00E-15	1.50E-03	2.60E-04	6.00E-04	4.90E-12	0.02
	Gene expression n BC[3] (compared with normal tissue)	down	down	down	down	down	up
	Normal tissue vs ER+ BC[5] [4]	2.20E-13	0.01	1.30E-03	4.20E-03	3.30E-12	0.05
	Gene expression in ER+ BC (compared with normal tissue)	down	down	down	down	down	up
	Normal tissue vs ER- BC[6] [4]	1.10E-04	0.03	0.01	0.01	0.02	0.05
	Gene expression in ER- BC (compared with normal tissue)	down	down	down	down	down	up
	DCIS vs invasive BC[3] [4]	0.04	0.12	0.01	0.66	0.24	0.001
	Gene expression in invasive (compared with DCIS)	down	-	up	-	-	up

1) Q-value from SAM of gene expression in normal breast tissue according to serum estradiol
2) s-est = serum estradiol
3) BC = breast cancer
4) P-value from two-sided t-test
5) ER+ BC = estrogen recepor positive breast cancer (n=53)
6) ER- BC = estrogen recepor negative breast cancer (n=8)

Table 2 Genes independently associated with serum estradiol in a linear regression modell. All genes differentially expressed according to serum estradiol (Table 1) were included. Values shown are corrected for age, menopause and current hormone therapy. After leave-one-out stepwise selection the following covariates remained:

Covariate	Estimate[1)]	Std error	p-value
SCGB3A1	0.068	0.025	0.009
TLN2	0.142	0.061	0.024
PTGS1	-0.145	0.066	0.030
SLITRK4	0.086	0.075	0.25[2)]
RSPO1	0.045	0.045	0.32[2)]
DCLK1	0.023	0.063	0.71[2)]

1) Estimate denotes the beta-value corresponding to each covariate in the regression equation.
2) Values for the non-significant genes are from the last model before they were excluded.

Table 3 Serum hormones independently associated with mammographic density in linear regression models. Values shown are corrected for age, HT and BMI. Through leave-one-out stepwise elimination of covariates, prolactin, SHBG and testosterone were excluded and the following variables remained.

	Absolute density		Percent density	
Covariate	Estimate[1)]	p-value	Estimate	p-value
Parity	-8.18	0.01	-	-
Serum estradiol	95.55	7.1E-05	51.31	9.3E-03

1) Estimate denotes the beta-value corresponding to each covariate in the regression equation.

Table 4 Genes significantly differentially expressed according to serum estradiol levels in breast carcinomas. A) Quantitativ SAM analysis for differential expression according to serum estradiol with q-values and direction of regulation indicated. B) Significance testing of difference in gene expression of the genes identified in A) in different sample cohorts.

	Gene Name	*AREG*	*GREB1*	*TFF3*	*TFF3*	*TFF1*
A	Chromosomal location	4q13-21	2p25.1	21q22.3	21q22.3	21q22.3
	q-value (%) SAM all tumors[1)]	0	0	20.5	20.5	20.5
	Gene expression in high s-est (compared with low s-est)[2)]	up	up	up	up	up
	q-value (%) SAM ER+ BC[1)3)]	0	0	-	-	-
	Gene expression in high s-est (compared with low s-est)[2)]	up	up	-	-	-
B	BC[4)] vs normal breast tissue[5]	0.38	0.18	4.80E-05	2.60E-04	1.20E-07
	Gene expression in BC[4)] (compared with normal)	-	-	up	up	up
	ER+ vs ER- BC[4)5]	0.08	4.80E-08	2.00E-06	2.40E-07	0.002
	Gene expression in ER+ BC (compared with ER- BC[6)])	up	up	up	up	up

1) Q-value from SAM of gene expression according to serum estradiol
2) Gene expression in samples from patients with high compared with low serum estradiol
3) ER+ BC = Estrogen receptor positive breast cancer (n=53)
4) BC = breast cancer
5) P-value from two-sided t-test
6) ER- BC = Estrogen receptor negative breast cancer (n=8)

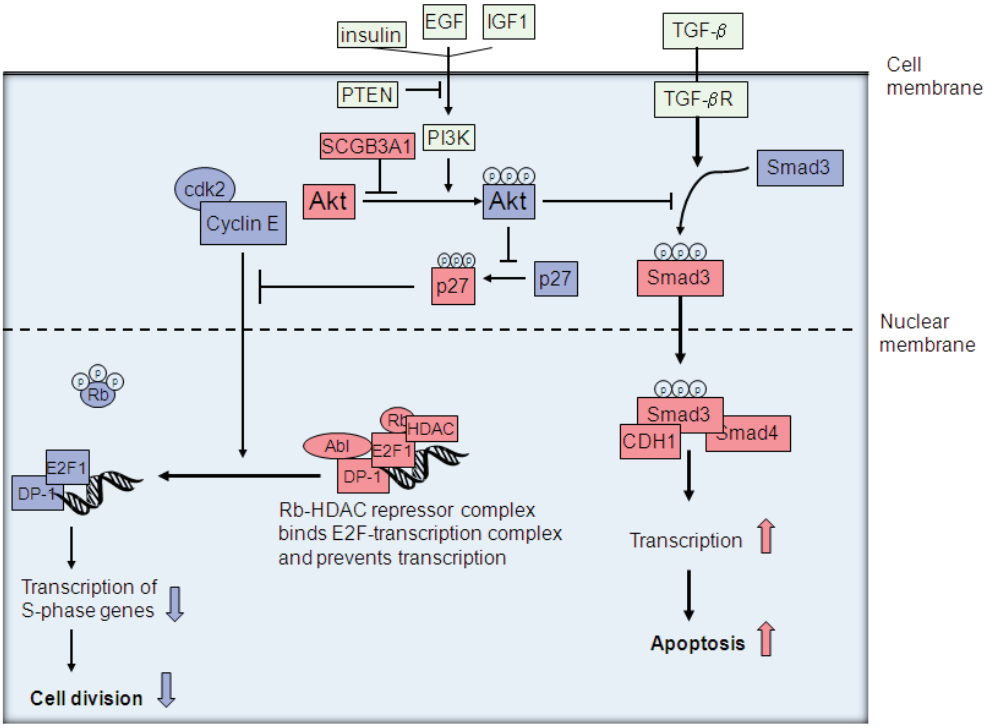

Figure 1 Simplified illustration of the cellular mechanisms of SCGB3A1. SCGB3A1 inhibits the phosphorylation of Akt leading to reduced cell cycle division and increased apoptosis. Molecules in red are increased/stimulated as result of SCGB3A1-action, whereas molecules in blue are decreased/inhibited.

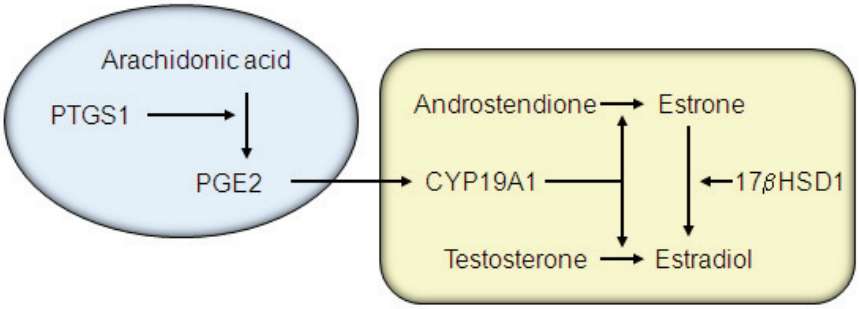

Figure 2 Schematic illustration of mechanism of PTGS1. PTGS1 induces PGE2-production. PGE2 increases the expression of aromatase (CYP19A1) which in turn converts androgens to estrogens in adipose tissue. 17βHSD1= 17bhydroxysteroid dehydrogenase.

Supplementary tables and figures

Table S1: Criteria for estimation of menopausal status.

FSH>20	LH>15	FSH>LH	s-est[1] <0.1	menopausal status	criteria
1	1	1	1	post	
1	1	1	0	post	s-est<0.3
1	1	1	0	peri	s-est>0.3
1	0	1	1	post	
1	1	0	0	peri	s-est>0.3 without HT[2]
1	1	0	0	post	s-est <0.3 or HT
1	0	1	0	post	s-est >0.3 without HT
1	0	1	0	peri	s-est <0.3 or HT
0	0	1	1	peri	FSH>15
0	0	1	1	pre	FSH<15
0	1	0	0	pre	Age>50=peri
0	0	1	0	peri	s-est <0.3
0	0	1	0	pre	s-est >0.3
0	0	0	0	pre	
Any	Any	Any	Any	post/peri	HT-use

1) serum estradiol
2) HT: hormone therapy

Table S2: Genes differentially expressed according to serum estradiol in breast carciomas and their expression in normal breast tissue

	AREG	***GREB1***	***TFF3***	***TFF3***	***TFF1***
Agilent ID	A_23_P259071	A_23_P329768	A_23_P257296	A_23_P393099	A_24_P322771
p-value (normal vs tumor)[1]	0.38	0.18	4.8E-05	2.6E-04	1.2E-07
Mean normal	-0.35	-1.29	1.45	1.44	-2.76
Mean tumor	-0.61	-1.02	2.74	2.65	-1.13
p-value (ER+ vs ER-)[1]	0.08	4.8E-08	2.0E-06	2.4E-07	2.3E-03
Mean ER+ tumors (n=53)	-0.44	-0.60	3.21	3.19	-0.81
Mean ER- tumors (n=8)	-1.54	-3.17	-0.17	-0.51	-3.32
q-value (%) SAM[2]	52.9	28.4	52.9	39.5	75.3

1) Two-sided t-test
2) Q-value for genes up-regulated in samples from women with high serum estradiol (SAM on samples from normal breast tissue according to serum estradiol).

Table S3: Genes differentially expressed according to estradiol treatment in Wilson et al and according to serum estradiol in the current study

Genes differentially expressed between mice treated and not treated with estradiol in Wilson et al, 2006		Quantitativ SAM according to serum estradiol levels in the current study. Gene expression in high serum estradiol.		
Symbol	gene experession in estradiol-treated mice	Normal breast tisssue	Breast carcinomas	ER+ breast carcinomas
TFF1	up	up	up	-
MYBPC1	up	up	-	-
AREG	up	up	up	up
SCGB2D2	up	-	-	-
TFF3	up	up	up	-
SCGB2A2	up	-	-	-
GREB1	up	up	up	up
SERPINA1	up	up	-	-
C1orf34	up	up	-	-
PIP	up	-	down	down
AGR2	up	-	-	down
SERPINA3	up	up	-	-
PRR4	up	-	-	-
HBE1	up	-	-	-
EEF1A2	up	-	-	-
DSU	up	-	-	-
MYB	up	-	-	-
AZGP1	up	-	-	down
TACSTD1	up	-	-	down
KRT19	up	-	down	down
CELSR2	up	-	-	-
FXYD3	up	-	-	-
XBP1	up	-	-	down
PRG4	up	-	down	down
MMP	up	-	-	-
MMP12	down	-	down	down
ME1	down	up	down	down
CXCL11	down	-	-	-
COL6A	down	-	-	-
CXCL11	down	-	-	-
GATA3	down	down	-	-
HSPG2	down	-	-	-
CCl2	down	-	down	down
EMILIN	down	-	-	-
CXCL10	down	-	-	-

Tabls S3 cont

Genes differentially expressed between mice treated and not treated with estradiol in Wilson et al, 2006		Quantitativ SAM according to serum estradiol levels. Gene expression in high serum estradiol -		
Symbol	gene expression in treated mice	Normal breast tisssue	Breast cancers	ER+ breast cancers
RARRES1	down	up	down	-
S100A8	down	-	down	down
IGJ	down	up	-	-
CXCL9	down	-	down	down
FN1	down	-	-	-
DPT	down	-	down	-
RGS5	down	-	down	down
CCL19	down	-	down	down
DPT	down	-	down	-
TPSAB1	down	-	-	-
CSPG2	down	up	-	-
ENPEP	down	-	-	-
SERPINH1	down	up	-	-
RGS5	down	-	down	down
IL8	down	-	-	-
IGHA1	down	-	down	-
BGN	down	-	-	-

Paper IV

Identification of SNP markers with putative influence on mammographic density and breast cancer risk